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Mouse Models of Neuroanatomical, Behavioral, and Genetic Correlates of Language-related Impairments: an Investigation of Core Phenotypes.

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The development of the brain is an immensely complicated process that is highly regulated by numerous genetic pathways. Disruption within any stage of this operation—from proliferation, to neuronal migration, to synaptogenesis—could ultimately lead to deleterious behavioral outcomes, ranging from severe intellectual disability and gross motor developmental delays to more subtle cognitive impairments such as language disability. It is therefore no surprise that neuroanatomical anomalies resulting from of altered cortical development are associated with a number of language related neurodevelopmental disorders such as specific language impairment (SLI), dyslexia, and autism spectrum disorders (ASD). Over the past decade, genetic association studies have identified numerous genetic variants that are more commonly observed in language and reading impaired populations. However, these studies cannot ascertain whether these genes are central to the etiology of the disorder (since association but not causatlity is repoterd). As another approach, rodent models have provided an invaluable tool to elucidate functional gene-brain-behavior relationships that are nearly impossible to examine clinically. Indeed, rodent models in the past have been successful in linking clinically relevant neuropathological changes associated with language disability to poor behavioral outcomes in the domains of auditory processing and cognitive performance on water maze tasks—behaviors that have been shown to be deficient in language and

reading impaired populations. Given the pressing need to understand how genetics underlie the development of language dysfunction, the series of studies presented in this thesis was designed to examine and characterize neurobehavioral, neuroanatomic, and genetic profiles of language and reading related disorders in rodents, specifically using recombinant inbred and transgenic strains of mice. In these experiments, we defined relationships between: 1) key behavioral phenotypes associated with language dysfunction and social communication/interaction; 2) disruption of neuronal migration; 3) changes in subcortical anatomy along the central auditory pathway; and 4) sex differences across these factors. From these relationships, our goal was to identify how promising risk genes identified from clinical populations (e.g., *DCDC2* (dyslexia) and *CNTNAP2* (SLI and ASD)) as well as associated neuroanatomical anomalies of neuronal migration, may collectively mediate the constellation of language-related and cognitive dysfunctions observed in relevant clinical populations.

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CHAPTER 1

Introduction

1.1 Language disability and related neurodevelopmental disorders

The ability to produce and understand language is an exceptionally complex process involving a variety of reciprocal behavioral mechanisms, from processing and learning the acoustic features of speech, to figuring out how to map them onto articulatory gestures, and ultimately integrating those features to produce meaningful speech (Pennington & Bishop, 2009). It is no surprise that early disturbances to any component of this network during development may lead to a variety of core impairments that disrupt the language trajectory.

One such resulting disorder is specific language impairment (SLI), which is a developmental communication disorder characterized by a profound delay in the acquisition and/or development of normal language in the absence of other medical causes (i.e., hearing impairment, impaired motor articulators, neurological injury) or generalized cognitive impairments. In the realm of research, SLI can be used as an umbrella term to describe a number of speech and language disorders including expressive language disorder (i.e., verbal dyspraxia), mixed receptive-expressive language disorder, and phonological disorder (Li & Bartlett, 2012; Newbury & Monaco, 2010). SLI has an overall prevalence of 5%–8% among pre-school aged children (Harel et al., 1996; Tomblin et al., 1997), and interestingly, symptoms associated with SLI are frequently also observed in other developmental disorders such as dyslexia (Newbury et al., 2011) and autism spectrum disorders (ASD; see Bishop, 2010, for review).

There is also a significant comorbidity between SLI and developmental dyslexia—a neurobehavioral learning disorder characterized by a fundamental and specific deficit in reading and spelling ability despite adequate intelligence, educational opportunity, and/or socioeconomic status. It is estimated that 43% of children with SLI will later be diagnosed with dyslexia, and 55% of children with dyslexia show symptomatology overlapping with SLI (McArthur et al., 2000; Snowling, Bishop & Stothard, 2000), suggesting some shared etiology. In addition, both SLI and dyslexia typically present with a general impairment in phonological processing—that is, the ability to discriminate the relationship and differences between phonemes (speech sounds)—and this phonological deficit is widely supported and accepted as a potentially causal factor in disrupted language and (ultimately) reading ability (see Pennington & Bishop, 2009, for review). Disordered phonological processing may in turn be mediated by underlying deficits in basic neural processes such as rapid auditory processing (RAP), working memory, and/or attention. However, like SLI, a diagnosis of dyslexia is only applicable in the absence of other clinically causal conditions such as hearing impairment or intellectual disability.

Autism spectrum disorder (ASD) is another neurobehavioral developmental disorder characterized by impairments in three behavioral domains, with onset by age three: 1) social behavior; 2) language development; and 3) repetitive behaviors and restricted interests. However, clinical manifestation varies greatly within and across of each these domains, with substantial phenotypic and genetic heterogeneity (American Psychiatric Association, 2000). Language impairment is, however, a core symptom of ASD, and may manifest in several different ways, including (but not limited to) delays in

language onset, impairments in structural aspects of language (phonological), or impairments in language usage (pragmatics; Bishop, 2010). Based on diagnostic criteria, SLI and ASD cannot present comorbidly, however, language deficits observed in each respective population show striking similarity. In addition, family studies investigating probands have shown familial clustering with SLI and ASD, further suggesting that there may be some overlapping genetic etiology between the two clinically distinct disorders (Bartlett et al., 2012).

Overall, despite the variability in clinical diagnosis within and across neurodevelopmental disorders such as SLI, dyslexia, and ASD, these disorders do share overlapping symptomatology in the domain of language impairment. It may be that these similarities could point to shared biological and/or genetic factors that increase risk of language-related deficits, and may explain comorbidity often observed between SLI and dyslexia, as well as familial aggregation between SLI and ASD (Alcántara et al., 2012; Bartlett et al., 2012; Benasich et al., 2006; Bhatara et al., 2013; Bishop, 2003; Bishop, 2010; Boscariol et al., 2010a; McArthur & Bishop, 2005; Pennington & Bishop, 2009). These shared core symptoms that are associated with language learning and reading ability are considered “intermediate phenotypes” (or endophenotypes) of language ability, and may be used to help researchers study complex neurobehavioral disorders such as SLI, dyslexia, and ASD using a more homogenous population (Grigorenko, 2009). The examination of clinical populations based on its component “parts” has greatly aided clinical researches in identifying genetic variants associated with and across numerous intermediate phenotypes related to clinical disorders, as opposed to performing association studies on clinical diagnosis alone (see Carrion-Castillo, Franke & Fisher,

2013 and Graham & Fisher, 2013, for review). To date, a specific neurobiological cause for language and/or reading impairment has not yet been identified, but human genetic and neurobiological studies have provided a platform for more fine-grained investigation of potential gene, brain, and behavior relationships through the use of rodent and other biological models, where ongoing experimentation can utilize intermediate phenotypes to better understand the biological etiology of a disorder.

1.2 Sex differences in the incidence of language disability and related disorders

Clinically, males seem to be at a general behavioral disadvantage based on evidence showing that males are more commonly diagnosed with neurodevelopmental disorders including cerebral palsy (Odding et al., 2006), attention deficit hyperactivity disorder (Faraone et al., 2003), ASD (Whiteley et al., 2010), SLI (Broomfield & Dodd, 2004), and dyslexia (Rutter et al., 2004). With regard to neurobehavioral disorders related specifically to language impairment (e.g., SLI, dyslexia, and ASD), males are more often diagnosed relative to females, with an odds ratio (male:female) of 3:1 for SLI (Broomfield & Dodd, 2004); 3.3:1 for dyslexia (Rutter et al., 2004); and 6.5:1 for ASD (Whiteley et al., 2010). Although the etiology of this sex difference is still unknown, evidence does support sex differences in how typical (non-language impaired) males and females process speech/language. For example, fMRI data shows that females have stronger activation of cortical language areas when processing language, and this difference is seen throughout development (Burman, Bitan & Booth, 2008). In addition, adult females show bilateral activation of neural regions while performing a phonological task, whereas activation patterns in adult males are generally isolated to left hemisphere (Shaywitz et al., 1995). A recent study has also reported sex differences in the

physiological (auditory brainstem) response to a stop-consonant speech syllable, indicating that the neural response in males and females differed only for rapidly changing components of the speech stimulus, but not during the slower changing parts (Krizman, Skoe & Kraus, 2013). During these time points of behavioral divergence, females showed earlier peaks in response to speech sounds relative to males, indicating that females may be more efficient processors of auditory information even at the level of the brainstem. The female advantage in language ability in both neural activation and bilateral organization may suggest that a greater biological or genetic insult may be necessary to elicit language impairment in females as compared to males. Despite this evidence of sex differences in language and auditory processing research from typically developing males and females, the underlying biological and genetic mechanisms mediating sex differences in language disorder prevalence remain unclear. However, empirical examination of sex differences in a rodent model of malformations of cortical development (MCD) may help elucidate this issue (addressed below).

1.3 Auditory processing—one of many intermediate phenotypes of language and reading related disability

Deficits in auditory temporal processing are a common phenotype shared across SLI, dyslexia, and autism, suggesting that auditory processing may be a fundamental skill in the development of language, as well as future reading ability. One of the first studies to identify a link between impaired rapid auditory processing (RAP) behavior and language disability in children was conducted by Tallal & Piercy, (1973). These researchers found that children diagnosed with language disability had difficulties identifying differences in tone sequences presented in rapid succession (< 305 ms). However, when temporal

intervals for stimulus changes were longer, language impaired children were capable of performing the two-tone discrimination task at the level of matched typically developing controls (Tallal & Piercy, 1973). Subsequent studies have further supported this association in other reading and language disabled populations, where difficulties in responding to quickly changing verbal and nonverbal auditory stimuli are also seen (Alcántara et al., 2012; Boscariol et al., 2010a; Cohen-Mimran & Sapir, 2007; Tallal & Piercy, 1973; Tallal, Miller & Fitch, 1993; Tallal & Newcombe, 1978; Vandermosten et al., 2011). Furthermore, studies using infants (6–12 months) as a "pre-lingual" model to study language development have been successful in predicting future language learning impairment at 3 years of age based on evidence of deficient RAP at 7.5 months old (Benasich & Tallal, 2002; Benasich et al., 2006; Choudhury et al., 2007). These long term predictive associations are also seen for early EEG measures during processing of rapidly changing acoustic information (Choudhury & Benasich, 2011).

In ASD, evidence of atypical auditory processing behavior is also reported. Enhanced perceptual abilities are seen in simple pitch discrimination, identification, and memory. In the ASD literature, these tasks are suggested to tap "local" acoustic processing, where individuals process information based on component (details) of a whole (see O'Connor, 2012 and Ouimet et al., 2012, for review). This advantage in local pitch discrimination was even seen at rapid stimulus presentation rates. However, when asked to perform an auditory processing task that utilized "global" acoustic cues (processing a complex gestalt), impairments in auditory processing behaviors were elicited. These tasks where deficient auditory processing behaviors are observed in ASD individuals include detecting speech in noise (Alcántara et al., 2004; Alcántara et al.,

2012), silent gaps in noise (Bhatara et al., 2013), and discriminating between competing words in sentences (i.e., trying to isolate conversations within a social gathering with many conversations; DePape et al., 2012).

Although findings supporting a central auditory processing deficit in reading and language impaired individuals remain controversial (Snowling & Hulme, 2012), the theory (described in a very simplistic manner) purports that intact low-level auditory processing ability across both temporal and frequency dimensions is needed for early language learners to effectively develop phonological skills, allowing fine-grained acoustic features related to speech to be mapped onto phonemes (basic units of language to convey differences in meaning; (Pennington & Bishop, 2009). In turn, phonemic awareness allows learners to ultimately map phonemes to graphemes (units of written language that make up a phoneme), an essential skill for adequate reading behaviors (Pennington & Bishop, 2009).

It must be noted that impairments in RAP are one of many fundamental intermediate phenotypes of language and reading disability. Other cognitive domains have been implicated including, but not limited to, short term and working memory, as well as other sensory processing deficits (e.g., motor, tactile, visual, and multi-modal; Archibald & Gathercole, 2006; Baddeley, 2003; Briscoe & Rankin, 2009; Grant et al., 1999; Laasonen, Service & Virsu, 2001; Laasonen, Service & Virsu, 2002; Livingstone et al., 1991; Montgomery, Magimairaj & Finney, 2010; Montgomery, 2002; Stoodley et al., 2000; Zhang et al., 2005). Although the behavioral and biological etiology of language dysfunction remains unclear, the heterogenous nature of critical underlying functions

highlight the issue that language disorders are complex in etiology, and that failure in one or more of these low-level pre-linguistic systems can lead to future language problems.

1.4 Malformations of cortical development (MCD)—a potential neuroanatomical substrate of language and reading disability

Language development, biologically, is a complex process, involving concerted neural systems that integrate auditory, visual, somatosensory, and memory functions in order for the individual to perceive and produce language. Early neurological disturbances in any of these networks could lead to a variety of impairments that ultimately lead to language disability. Thus, it is no surprise that associations between language-related neurodevelopmental disorders and neurological dysfunction related to cortical development have been made. A study conducted by Galaburda and colleagues (1985) was one of the first to report a compelling dataset suggesting that focal malformations of cortex (reflecting disruptions during early neuron migration) may play a pathogenic role in the development of dyslexia. Specifically, *post mortem* examination of brain tissue obtained from four males previously diagnosed with dyslexia revealed an asymmetrical distribution of cortical anomalies predominantly in the left hemisphere, particularly clustered around inferior frontal and superior temporal regions (areas related to language and reading ability). These cortical malformations were consistent with disrupted neuronal migration, revealing pockets of ectopic neurons in layer 1, as well as underlying cortical dysplasias including micropolygria. Subsequent investigation of *post mortem* histological brain tissue of three female dyslexics found a similar pattern of atypical cortical anomalies in dyslexic male brains, including molecular layer ectopias and cortical dysplasia (though to a quantifiably lesser extent; Humphreys, Kaufmann &

Galaburda, 1990). Further, *post mortem* cytroarchitectonic analysis of dyslexic brains also revealed more small (and fewer large) neurons in the auditory nucleus of the thalamus (medial geniculate nucleus; MGN)—a finding of particular of interest, given that deficits in RAP are seen in reading and language disabled populations (reviewed above; Galaburda, Menard & Rosen, 1994). Following the hallmark series of studies conducted by Galaburda and colleagues (Galaburda et al., 1985; Galaburda, Menard & Rosen, 1994; Humphreys, Kaufmann & Galaburda, 1990) and the advent of more sophisticated *in vivo* structural imaging, cortical anomalies related to potential disturbances in early neurological development have been found more recently in dyslexics and language impaired populations, including reductions in cortical grey matter (Brambati et al., 2004; Clark & Plante, 1998; Evans et al., 2014; Kronbichler et al., 2008), atypical white matter organization and callosal morphology (Chang et al., 2007; Darki et al., 2012; Hasan et al., 2012; Klingberg et al., 2000; Niogi & McCandliss, 2006; Waiter et al., 2005), periventricular nodular heterotopia (Chang et al., 2005; Christodoulou et al., 2013; Wegiel et al., 2010), and polymicrogyria (Boscariol et al., 2009).

It may be that subtle cortical anomalies within regions surrounding the perisylvian area can lead directly to disorganized/aberrant connectivity among neural networks subserving language and reading relevant processes. However, it is also possible that regions distal to malformations may be aberrantly affected over development, as evidenced by animal work in non-human primates showing that prenatal frontal lesions produced cytroarchitectonic disruptions in temporal and occipital regions, while postnatal lesions showed changes in the medial dorsal nucleus of the thalamus (thalamic nucleus

that relays information to the frontal cortex; Goldman & Galkin, 1978; Goldman, 1979). Unfortunately, the histological samples collected by Galaburda et al, (1985), cannot provide evidence of cortical rewiring within the four dyslexic brains, but this study was one of the first to provide a link between neuroanatomical malformations and reading and language disability. The series of histological studies showing a relationship between cortical anomalies and disrupted reading and language development has prompted the examination of rodent models to further explore this link.

1.5 Animal models of MCD—a link between cortical anatomy and auditory processing

1.5.1 Injury-induced microgyria

Histopathological findings in dyslexic individuals prompted the development of animal models to further study the behavioral and neuroanatomical consequences of disrupted cortical development. Various neurobehavioral assessments were designed specifically to model intermediate phenotypes of language and reading disability in rodents, and these have been successful in creating a behavioral profile in support of a potentially functional role between MCD and reading and language-related behaviors. One such model developed by our research group examined injury-induced lesions that disrupted late stages of cortical neuronal migration in newborn rats (Rosen et al., 1992). Small freezing probes placed directly on the skull of postnatal day 1 (P1) rat pups resulted in partial or complete necrosis of cells directly underlying the placement of the probe, ultimately resulting in the formation of a 4-layered microgyrus surprisingly similar in structure to that of cortical microgyria previously observed in dyslexic brains (Galaburda et al.,

1985). Further neuroanatomical analysis of male rats with injury-induced microgyria (in parietal cortex) also revealed changes in both ventral posterior nucleus of the thalamus (VPN; thalamic nucleus with direct relay to somatosensory cortex) as well as MGN neuronal morphology (Herman et al., 1997; Rosen et al., 2006). Specifically, more small than large neurons was seen in the VPN and MGN of microgyric rats in comparison to sham controls (along with fewer large neurons). Follow-up neuroatomical tracing studies in injury-induced rat models of cerebrocortical microgyria also found widespread alterations in efferent and afferent neural connectivity between regions of microgyric malformation and homotopic (uninjured) cortex, as well as disruptions in thalamo-cortico/cortico-thalamic projections between the VPN and cortical regions surrounding the microgyria (Rosen, Burstein & Galaburda, 2000). Furthermore, induced microgyria in the postero-medial barrel subfield also led to diffuse changes in contralateral barrel fields, resulting in increased total area but no change in the number of individual barrel regions, suggesting further changes in callosal and/or thalamic connectivity (Rosen, Windzio & Galaburda, 2001).

Behavioral examination of male rats with injury-induced cerebrocortical microgyria in the region of parietal cortex also revealed impairments in RAP on various auditory processing paradigms, including a go/no-go operantly conditioned target discrimination task (Fitch et al., 1997; Fitch et al., 1994), two tone odd-ball (OB) task (Clark et al., 2000a; Clark et al., 2000), as well as a silent gap detection paradigm (Peiffer, Rosen & Fitch, 2004; Peiffer et al., 2004). Across all tasks, male microgryic rats showed impaired auditory processing performance specifically for short (rapid) duration stimuli, but when task parameters included longer stimulus presentations, performance

was comparable to matched controls. Fascinatingly, the pattern of auditory processing behavior in male microgyric rats paralleled observations made in language impaired populations, specifically with regard to deficits in processing of rapid/short but not slow/long stimuli.

1.5.2 Spontaneously occurring ectopias—NZB/BINJ and BXSB/MpJ mice

Certain inbred strains of mice, such as NZB/BINJ and BXSB/MpJ, exhibit spontaneously occurring clusters of mismigrated neurons in layer 1 of the cortex—a structural "ectopia" phenotype histologically similar to those identified in *post mortem* analysis of human dyslexic brains (Sherman et al., 1987; Sherman, Galaburda & Geschwind, 1985). In these mice, ectopic malformations present in 40–60% of cases, and appear prenatally (suggesting that dysfunction of neuronal migration may be the cause). Interestingly, preliminary genetic analysis of NZB/BINJ mice suggest that occurrence of ectopias follows an autosomal recessive pattern of transmission with incomplete penetrance, but no particular gene has been implicated in the predisposition for this phenotype (Sherman et al., 1994). Histological analysis of NZB/BINJ and BXSB/MpJ mice revealed disruptions in cortical lamination underlying the neural ectopias (Sherman et al., 1990), as well as aberrant connectivity to regions of the cortex and the thalamus (Jenner, Galaburda & Sherman, 2000). Electrophysiological characterization of cortical ectopias identified several different neuronal subtypes within the ectopic collections, including neurons receiving both inhibitory and excitatory synaptic input from surrounding normatopic cortex (Gabel & LoTurco, 2001; Gabel, 2011). Furthermore, ectopic neurons showed hyper-excitability and generalized epileptiform activity, which was also seen in non-ectopic surrounding cortex (Gabel & LoTurco, 2001).

Based on the widespread cortical dysfunction observed histopathologically and electrophysiologically, it is no surprise that behavioral characterization of both NZB/BINJ and BXSB/MpJ revealed a number of cognitive and memory impairments (and occasional superior proficiency) across spatial and non-spatial maze paradigms (Boehm et al., 1996a; Boehm et al., 1996b; Hyde, Hoplight & Denenberg, 1998; Hyde et al., 2000a; Hyde et al., 2000b; Hyde et al., 2001; Schrott et al., 1992; Schrott et al., 1993). Deficient RAP of embedded tones was also observed in NZB/BINJ and BXSB/MpJ mice through assessment on a modified startle reduction paradigm (Clark et al., 2000b; Peiffer et al., 2001; Peiffer, Rosen & Fitch, 2002), as well as auditory event related potentials (AERP; Frenkel et al., 2000; Peiffer et al., 2001).

1.5.3 RNA interference—various neuronal migration anomalies related to genetic manipulation of candidate dyslexia susceptibility genes.

The use of RNA inference (RNAi) has helped to advance the study of behavioral genetics by providing a means to manipulate genetic function *in vivo*, and also to manipulate spatial and timing specificity of genetic disruption. These emergent techniques have allowed researchers to more precisely examine potential functional relationships between gene, brain, and behavior in neurodevelopmental disorders. Within our research group, we have utilized the RNAi model in rats to examine how reductions in gene expression of clinically identified candidate dyslexia susceptibility genes (CDSGs) may affect neurodevelopment and behavior, ultimately to inform any potential etiological mechanisms of language and reading disability. Functional *in vivo* studies involving RNAi knockdown of rodent homologs of CDSGs have found that some CDSGs may play a role in neuronal migration. For example, RNAi of *Dcdc2*, *Dyx1c1*, and *Kiaa0319*,

respectively (all rodent homologs to human CDSGs), leads to delayed and/or disrupted neuronal migration 4–7 days post-transfection (Burbridge et al., 2008; Paracchini et al., 2006; Wang et al., 2006). In the adult brain, delays in neuronal migration in *Dcdc2*, *Dyx1c1*, and *Kiaa0319* RNAi transfected rats manifest in white matter heterotopia, hippocampal dysplasias, and cortical ectopias (unrelated to injection site; Burbridge et al., 2008; Paracchini et al., 2006; Rosen et al., 2007). Additional histological analysis of the MGN in *Dyx1c1* RNAi transfected rats found more small cells in the MGN than large cells—a pattern of anomalies similar to those observed in male microgyric rats, as well as human dyslexic brains (Szalkowski et al., 2013). RAP impairments in have also been observed in rats embryonically transfected with *Dyx1c1* and *Kiaa0319* (Szalkowski et al., 2013; Szalkowski et al., 2012; Threlkeld et al., 2007).

1.5.4 Summary of animal models of MCD and auditory processing behavior

Together, the series of neuroanatomical, behavioral, and electrophysiological characterizations in rodents across models of injury-induced microgyria, spontaneously occurring cortical ectopias, and RNAi manipulation of genes associated with neuronal migration provide an extensive and fascinating data-set. Each of these models present with cortical malformations histologically similar to those observed in *post mortem* human dyslexic brains, and further neuroanatomical and electrophysiological findings in these rodent models reflect wide-spread neuropathological changes of cellular morphology in structures immediate and distal to the malformations, as well as aberrant projects to and from the malformations and surrounding cortex. Even more exciting is that rodent models of MCD and aberrant neural connectivity co-occur with impaired RAP behavior, but current data so far cannot definitely claim that auditory processing

impairments are a direct result of MCD. Despite this, MCD in specific regions of the cortex could be a biomarker predicting future language/reading impairment.

Although these models are more directly related to neuroanatomical anomalies found in dyslexic brains, the bigger picture obtained from these data tell a story of diffuse reorganization of the developing brain as a result of disruption in neuronal migration resulting in subtle MCD. Given that *in vivo* structural imaging provides evidence of MCD in both SLI and ASD, the rodent and human histological and behavioral studies reviewed above appear to be relevant to other reading and language related neurodevelopmental disorders, as well (Clark & Plante, 1998; Wegiel et al., 2010).

1.6 Sex differences in models of MCD

Behavioral and anatomical data on rodent models of MCD reviewed above focused primarily on male rodents, but extensive work examining sex differences has also been performed using these models, revealing an intriguing pattern of neuroanatomical and behavioral findings. In both injury models of microgyria (focal freeze lesion) in rats and spontaneously occurring ectopias (BXSB/MpJ) in mice, sex differences in RAP behavior was observed across auditory behavioral paradigms including a go/no-go operant two-tone discrimination paradigm, as well as a startle reduction paradigm utilizing a two-tone OB detection task (Fitch et al., 1997; Peiffer, Rosen & Fitch, 2002; Peiffer, Rosen & Fitch, 2004). As reviewed above, males with injury-induced or spontaneous malformations showed significant impairments in RAP, while injured or affected females with comparable resulting malformations showed significant discrimination at short duration conditions and their performance also did not differ significantly from sham

females (i.e., they showed no evidence of the impairment seen in males; Fitch et al., 1997; Peiffer, Rosen & Fitch, 2002; Peiffer, Rosen & Fitch, 2004).

Stereological examination of the MGN in female rats with injury-induced microgyric lesions also revealed no changes in MGN morphology—an intriguing finding, given that comparably injured males with similar malformations showed a significant shift toward more small neurons in the MGN as compared to controls (Herman et al., 1997; Rosen et al., 2006). However, comparison of MGN cell size distribution between male microgyric rats and female microgyric rats prenatally exposed to testosterone did show a similar pattern of more small neurons in the MGN as compared large (Rosen, Herman & Galaburda, 1999). These collective data suggest that testosterone may have a deleterious regulatory role or interaction with underlying reorganization of the MGN, and potentially the brain in general, either as a result of MCD or any insult to the developing brain (see Hill & Fitch, 2012, for review). However, behavioral outcomes following testosterone exposure in female microgyric rats has not been assessed using a RAP paradigm. However, a related model of early brain injury in rats has shown that exposure to testosterone concurrent to early brain injury in females lead to a more male-like behavioral profile, with impairments in RAP in testosterone-treated females where previously none were observed (Hill, Threlkeld & Fitch, 2011).

In contrast to injury models of disrupted neuronal migration leading to MCD, examination of sex differences in a genetic knockdown model of CDSG *Dyx1c1* showed that both male and female rats embryonically transfected with RNAi targeting against *Dyx1c1* showed similar impaired performance on complex (FM sweep and OB) RAP tasks (Szalkowski et al., 2013). Notably, neuroanatomical analysis of both male and

female *Dyx1c1* RNAi rats presented with MCD, and stereological analysis of MGN cell size in male rats, revealed a significant shift in cell size distribution toward more small cells—similar to the pattern observed in microgyric rats (Szalkowski et al., 2013). Although both clinical and injury based rodent models of MCD described reflect a female "protection" in RAP behavior, the genetic model examining *Dyx1c1* function provides a case where being "female" is not advantageous to behavioral outcome. However, it does highlight the need for rodent models that do not exhibit sex differences in behavior, to better understand the shared (or unshared) genetic and biological mechanisms ultimately leading to auditory processing dysfunction and (in humans) language impairment, as well as sex disparities in clinical incidence.

1.7 Genetics of language and reading disability

Before molecular genetic studies of language and reading disorders were possible, it was known that language disability typically clustered within families, and concordance rates among monozygotic twins were significantly higher than dyzygotic twins. Further epidemiological studies using genome-wide and targeted linkage analyses have identified a number of promising loci for dyslexia (*DYX1–9*) and SLI (*SLI1–4*). Further analysis of these regions using fine-grained mapping approaches and candidate gene studies, as well as utilization of behavioral intermediate phenotypes, have collectively led to the identification of putative risk genes including *DYX1C1*, *DCDC2*, *KIAA0319*, *ROBO1*, and *C2orf3/MRPL19* for dyslexia. In SLI, *CMIP* and *ATP2C2* have also been identified, but *CNTNAP2* may be another promising gene associated with language dysfunction/ability (see Carrion-Castillo, Franke & Fisher, 2013 and Graham & Fisher, 2013, for review). Below, a particular focus will be placed on *DCDC2* and *CNTNAP2*—

two genes implicated in reading and language disability, respectively, and both of which show great promise as potentially causal factors in the etiology of reading and/or language disability.

1.7.1 DCDC2—a candidate susceptibility gene for developmental dyslexia

Located on the most replicated dyslexia susceptibility locus, chromosome 6p22.3 (see Fisher & Francks, 2006, for review), *DCDC2* (doublecortin domain containing 2) was first implicated in developmental dyslexia by Meng et al. (2005) using a family based association study and linkage analysis focused on the region of chromosome 6p22. In this study, a number of gene variants located within *DCDC2* were identified as highly associated with poor composite reading scores. Subsequent studies using independent cohorts around the world have replicated these findings, identifying a number of gene variants throughout the *DCDC2* region, providing additional support for its role as a candidate susceptibility gene for dyslexia (Brkanac et al., 2007; Harold, 2006; Lind et al., 2010; Marino et al., 2011a; Marino et al., 2011b; Scerri et al., 2011; Schumacher et al., 2006). Furthermore, collective data has revealed that gene variants in *DCDC2* are associated with intermediate phenotypes of dyslexia including impairments in working memory (Berninger et al., 2008; Marino et al., 2011a; Marino et al., 2011b), attention (Couto et al., 2009), as well as phonology (Lind et al., 2010; Marino et al., 2011a; Marino et al., 2011b). Despite data clearly pointing to *DCDC2* as a strong susceptibility gene for dyslexia, the mechanisms through which *DCDC2* mediates these behaviors remain unclear. Nonetheless, some clues to its biological function have been offered through the use of animal models.

1.7.1.1 Animal model of Dyslexia: Dcdc2 RNAi and genetic knockout (KO) studies

DCDC2 is similar in structure to a well-studied neurodevelopmental gene, *DCX*, a key gene in the regulation of neuronal migration. To examine whether *DCDC2* plays a role in neuronal migration, Meng et al. (2005) embryonically transfected rats with shRNA targeted against *Dcdc2* (the rodent homolog of *DCDC2*) during the peak period of cortical neuronal migration. They found that neurons transfected with *Dcdc2* RNAi traveled a shorter distance from the ventricular zone in comparison to neurons transfected with the control plasmid (as measured four days following electroporation), suggesting that altered expression of *Dcdc2* led to an arrest in cellular migration. A subsequent study conducted by Burbridge et al. (2008) expanded on these findings by further characterizing the postnatal neurobiological outcome of rats with a knockdown of *Dcdc2*. Here, histological findings revealed neurons transfected with *Dcdc2* RNAi in a bimodal distribution, with unmigrated neurons clustered around the ventricular zone and underlying cortical white matter (periventricular heterotopia, PVH) and other labeled neurons over migrating past their target lamina. Overall, the atypical cortical morphology patterns seen in rat brains following embryonic knockdown of *Dcdc2* were similar to those found through *in vivo* imaging of reading disabled populations (Chang et al., 2005).

Additional neuroanatomical and biological studies of *Dcdc2* were conducted in a mouse KO (Wang et al., 2011). Here, surprisingly, no visible presence of gross neuromorphological anomalies as previously observed in rats embryonically transfected with *Dcdc2* RNAi (Burbridge et al., 2008). That is, structurally, the brains of mice with a systemic knockout of *Dcdc2* appeared similar to wild type mice, displaying no

migrational or laminar anomalies. However, electrophysiological examination in *Dcdc2* KO mice revealed alterations in neural physiological characteristics. In a study conducted by Che et al. (2013) increased resting membrane potential, increased spontaneous synaptic activity, and increased spontaneous glutamatergic synaptic activity was observed using whole-cell patch-clamp recordings on layer 2/3 pyramidal neurons in somatosensory cortex. Variations in temporal properties of action potential firing rates to the same repeated stimulus were also found in *Dcdc2* KO mice, suggesting an inability of neurons to exhibit a stable temporal firing pattern to a specific stimulus, potentially adding "noise" to the processing system. Overall, physiological evidence of *Dcdc2* function indicates a potential presynaptic mediation of neuronal activity as well as temporal firing patterns that may play a role in temporal encoding of sensory information.

Behavioral assessments using the mouse KO model of *Dcdc2* have also been performed. *Dcdc2* KO mice displayed unimpaired spontaneous locomotor and exploratory behavior on an open field task (Wang et al., 2011). They also were comparable to wild types in performing a simple configuration of the Hebb-Williams maze assessing basic visuo-spatial learning and memory (Wang et al., 2011). However, when the cognitive load of the task increased, mice with disrupted *Dcdc2* alleles (both heterozygous KO and homozygous KO) showed increased latency to complete the maze, even though their ability to acquire the task was unimpaired (as evidenced by comparable decreases in errors as well as distance to goal across trials in both *Dcdc2* mutants and wild type mice), thus suggesting a general impairment in visuo-spatial memory (Gabel et al., 2011). Additional assays found long-term memory impairment in both *Dcdc2* mouse mutants on a visual discrimination task, as well as increased anxious behavior on an

elevated plus maze in *Dcdc2* KO mice (Gabel et al., 2011). Collectively, the behavioral assessments conducted by Gabel et al. (2011) suggest that *Dcdc2* functions in mice to mediate more complex phenotypes, such as long-term and visual-spatial memory, that require a greater cognitive load in comparison to the more simple assessments conducted by Wang et al. (2011). In a clinical context, the behavioral data obtained from the mouse heterozygous and homozygous KO model of *Dcdc2* seems to correspond to findings that *DCDC2* may mediate working memory ability in humans for reading (Berninger et al., 2008; Marino et al., 2011b).

1.7.2 CNTNAP2—A potential candidate gene for SLI and ASD in language dysfunction

Biologically, *CNTNAP2* (contactin-associated protein-like 2; cellularly localized in Nodes of Ranvier to cluster K+ channels in myelinated neurons; Poliak et al., 1999) is of particular interest as a candidate gene for ASD because of its biological relation to the neurexin family—a family of proteins that mediate presynaptic development, and are highly associated with ASD (Sudhof, 2008). Further study of the *CNTNAP2* gene in epidemiological studies also suggests that it may mediate language learning endophenotypes (Whitehouse et al., 2011). Located on chromosome 7q35, *CNTNAP2* was first associated with language dysfunction in an Amish family with cortical dysplasia-focal epilepsy syndrome—a neuronal migration disorder characterized by epileptic seizures, language regression, intellectual disability, and autism (Strauss et al., 2006). Although this particular mutation was considered a rare recessive mutation, common variants of *CNTNAP2* have subsequently been implicated in language-related endophenotypes in autistic, dyslexic, and SLI populations (Alarcón et al., 2008; Newbury

et al., 2011; Peter et al., 2011; Rodenas-Cuadrado, Ho & Vernes, 2013; Vernes et al., 2008). Interestingly, variants of *CNTNAP2* have also been implicated in normal language variation (Whitehouse et al., 2011). Associated endophenotypes include age of language onset, nonword repetition scores, and expressive and receptive language abilities (see Rodenas-Cuadrado, Ho & Vernes, 2013, for review). In addition, *CNTNAP2* is of particular interest in language development due to its biological relationship with another well-known gene that had been identified as a causal gene in developmental speech and language disorder, FOXP2 (Vernes et al., 2008). Studies have found that FOXP2 directly binds to *CNTNAP2* to regulate its expression, creating an interesting connection between two genes that are both implicated in language function, and in turn suggesting a potential key molecular pathway for language development (Fisher & Scharff, 2009).

1.7.2.1 Animal model of SLI and ASD: Cntnap2 KO mice

In a study conducted by (Peñagarikano et al., 2011), extensive behavioral and neuroanatomical characterization of *Cntnap2* KO mice was conducted with a focus on intermediate phenotypes of ASD-like symptomatology. From this behavioral analysis, it was found that *Cntnap2* KO mouse pups made significantly fewer vocalizations in comparison to wild type controls when isolated from their mother. In adults, *Cntnap2* KO mice performed comparably to wild type controls on the Morris water maze, but a perseveration phenotype was observed when subjects were required to learn a new goal location (i.e., *Cntnap2* KO continued to swim to the previously learned location). Obsessive grooming behavior in *Cntnap2* KO mice was also observed, with both perseverative and obsessive behaviors suggesting analogy to the core repetitive and restrictive interest impairments observed clinically in ASD patients. In addition, a social

preference task was conducted and revealed that *Cntnap2* KO mice spent less time with an unfamiliar mouse in comparison to wild type controls (a sign of antisocial behavior), and instead were observed spending more time either grooming or digging—behaviors abnormal for the typically social C57BL/6J strain in the presence of a novel mouse. Again this parallels the abnormal social behavior core deficit in ASD populations.

Finally, neuroanatomical analysis of *Cntnap2* KO mice revealed evidence of disrupted neuronal migration (Peñagarikano et al., 2011). Specifically, immunohistological assessment found ectopic neurons located in the white matter, as well as evidence of late born neurons destined for superficial layers of the cortex in deep cortical layers (ectopic collections). Brains of *Cntnap2* KO mice also showed a reduced number of GABAergic interneurons in the cortex, with further assessment revealing that *Cntnap2* expression contributes to typical GABAergic (and dopaminergic) synaptic transmission. These findings support the neural asynchrony phenotype observed in the cortex of *Cntnap2* null mice.

1.8 Dissertation Purpose

The purpose of the current thesis was to examine and characterize neurobehavioral, neuroanatomic, and genetic profiles related to language and reading disorders in humans, but using rodent models. Specifically, the studies discussed here use recombinant inbred and transgenic strains of mice. We aimed to elucidate potential relationships between alterations in genetic expression, neural development, and behaviors associated with fundamental, non-verbal, aspects of language-learning and reading abilities that could reliably be assessed in a rodent model. These behaviors included RAP, working and

reference memory, and sensory integration. We aimed to use these assessments to specifically characterize the mouse homologs of the genes *DCDC2* and *CNTNAP2*, both of which have been highly implicated in dyslexia, SLI, and ASD, and have also been shown to be associated with various reading and language related measures within clinically defined populations. In addition, we aimed to define relationships between: 1) key behavioral phenotypes associated with language dysfunction and social ability; 2) disruption of neuronal migration (given that several different forms of neuronal migration anomalies have been implicated in various clinical populations with cognitive and social impairments, and have also been associated with *DCDC2* and *CNTNAP2*); 3) changes in subcortical anatomy along the central auditory pathway; and 4) sex differences across these factors. Our overall goal was to identify and understand how *DCDC2* and *CNTNAP2*, and associated neuroanatomical anomalies of neuronal migration, may collectively mediate the constellation of language-related and cognitive dysfunctions observed in relevant clinical populations with a genetic variant of one of those genes, or with a neuronal migration disorder of unknown genetic origin. Ultimately, we hope this data will contribute to the development of early biological screening techniques for neurodevelopmental and language-related disorders, as well as possible targeted behavioral therapies based on associated genetic/biological risk.

Extensive works from our research group as well as clinical literature have provided evidence that MCD co-occur with impairments in RAP behavior (Clark et al., 2000a; Clark et al., 2000b; Clark et al., 2000c; Fitch et al., 1994; Peiffer et al., 2001; Peiffer et al., 2004; Szalkowski et al., 2013; Szalkowski et al., 2012; Threlkeld et al., 2007). Interestingly, when female rodents with comparable injury and/or

neuroanatomical malformation were concurrently examined on these tasks, they actually showed normal RAP ability (i.e., comparable to female sham controls; Fitch et al., 1997; Peiffer, Rosen & Fitch, 2002; Peiffer, Rosen & Fitch, 2004). These findings could relate to the markedly lower prevalence of neurodevelopmental disorders among females. However, even though males are more commonly diagnosed with language-related neurodevelopmental disorders compared to females, it must not be ignored that a small subset of females do suffer from language and reading disability (Broomfield & Dodd, 2004; Rutter et al., 2004; Whiteley et al., 2010). Therefore it was important that an animal model was sought to further explore conditions in which females did *not* show a behavioral advantage (i.e., did show processing deficits). Fortuitously, a BXD29 mutant strain of mouse with a severe disrupted neuroanatomical phenotype was discovered, and initial behavioral characterization revealed a specific deficit in auditory temporal processing (Rosen et al., 2013). However, no other deficient behavioral phenotype was observed across sensorimotor and spatial learning and memory paradigms. Behavioral analysis was initially conducted in male mutant BXD29-*Tlr4*$^{lps\text{-}2J}$/J mice, only. Based on the male pattern of behavior reported in (Rosen et al., 2013), Chapter 2 was designed to replicate and extend the neurobehavioral profile of BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant mice to both males and females (who show comparable malformations), and specifically to investigate potential sex differences (i.e., whether BXD29-*Tlr4*$^{lps\text{-}2J}$/J females would display the same pattern of behavioral outcomes as their male BXD29-*Tlr4*$^{lps\text{-}2J}$/J counterparts). In addition to the replication of RAP, Morris water maze, and rotarod results, behavioral characterization also included an assessment of vocalization behavior in a social context as well as a social preference task. In males, results across RAP,

Morris water maze, and rotarod performance were replicated, with males showing a specific impairment in RAP. Assessment of female BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant mice surprisingly also showed a specific impairment in RAP comparable to that seen in mutant males. The presence of RAP impairments in BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant females provide an instance where female "protection" was not observed in the context of auditory processing behavior, and suggests that the severity of neuroanatomical phenotype in this model may contribute to penetrance of the impaired auditory processing phenotype even in females. Fascinatingly, sexually dimorphic behavior was observed on the Morris spatial water maze, with female BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant mice performing significantly *better* on the task, not only in comparison to female coisogenic controls, but also when compared to male mutants and controls. Examination of tasks designed to examine social behavior revealed that male BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant mice made significantly more vocalizations in the presence of female pheromones (female subjects were not assessed on this task due to a paucity of vocalization of behavior; Maggio & Whitney, 1985), and both male and female BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant mice spent more time socially interacting with a novel mouse instead of exploring an inanimate (novel) toy. Based on this social data, BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant mice (regardless of sex) seemed to show an atypical hyper-social phenotype, similar to that reported in Williams syndrome and several other rare neurodevelopmental conditions (e.g., Angelmans Syndrome; Fishman et al., 2011).

Given the severe impairments in auditory processing observed in both male and female BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant mice, Chapter 3 sought to determine if morphological changes in cellular subcortical anatomy within the central auditory pathway (including

MGN and ventral and dorsal subdivisions of the cochlear nucleus) were present. Stereological analysis found overall smaller neurons in the MGN of mutant mice, with cell size distribution revealing a shift toward more small and fewer large neurons. Results in the MGN were consistent across Sex. Interestingly, sexually dimorphic anomalies in the ventral and dorsal subdivisions of the cochlear nucleus (VCN and DCN, respectively) were observed, with female BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant mice showing a significant shift towards more large neurons in both the VCN and DCN. Based on the current data set, it remains unclear why female mutant mice present with more large cells in the cochlear nucleus since they do not show a preserved behavioral phenotype. Overall, results indicate a link between MCD, deficits in auditory processing, and changes in subcortical cellular morphology within the central auditory pathway.

In the clinical population, *DCDC2* shows great promise as a potential risk gene for developmental dyslexia. Based on these findings, additional characterization of *Dcdc2* KO mice was undertaken in Chapter 4 to expand behavioral assessment to previously unexamined intermediate phenotypes of language disability—including auditory processing, working and reference memory ability, sensorimotor processing, and temporal somatosensory processing. Examination of *Dcdc2* KO mice revealed a subtle auditory processing impairment using an embedded tone task. Results obtained from a 4/8 radial arm maze task measuring working and reference memory ability also revealed a generalized memory impairment in *Dcdc2* KO mice, as indexed by increased working and reference memory errors on the maze. No impairment in sensorimotor ability and learning was observed on the rotarod task, but a tactile discrimination impairment was found, with evidence that *Dcdc2* KO mice were unable to discriminate differences

between two tactile spatial frequencies (grades of sandpaper). Based on recent electrophysiological findings in *Dcdc2* KO mice, specifically demonstrating altered neuronal spike timing (Che, Girgenti & LoTurco, 2013), we suggest that the impaired behavioral phenotypes evidenced across auditory, memory, and somatosensory domains could be explained by deficient temporal encoding of sensory information at the level of the cortex, leading in turn to disrupted multi-modal neural representation of sensory stimuli.

CNTNAP2 is a gene of great interest in both the study of SLI and ASD. Given clinical evidence of co-occurring enhanced auditory perceptual abilities in relation to local pitch processing, coupled with difficulties in the perceptions of more spectro-temporally complex auditory information (see O'Connor, 2012 and Ouimet et al., 2012, for review), Chapter 5 sought to behaviorally characterize the auditory processing behavior of *Cntnap2* KO mice using a silent gap detection task, embedded tone detection task, and pitch discrimination task. Results showed impaired silent gap detection in *Cntnap2* KO mice, coupled with superior performance in *Cntnap2* KO subjects on embedded tone and pitch discrimination tasks when compared to wild type controls. This pattern of impaired and also enhanced auditory processing behavior in *Cntnap2* KO mice nicely parallels the behavioral findings observed in the clinical population, and provides support for a role in *Cntnap2* expression in mediating auditory processing behaviors in a rodent model.

Based on the intriguing pattern of abnormal auditory processing ability in *Cntnap2* mice, Chapter 6 aimed to examine whether changes in subcortical anatomy within the MGN, VCN, and DCN would be observed in these same mice. There was a

significant reduction in the number of neurons in the MGN of male *Cntnap2* KO mice. Stereological assessment of MGN cellular morphology also showed a shift toward more small than large cells in the MGN of male *Cntnap2* KO mice. Examination of VCN and DCN showed no changes in neuron cell count or cell size in *Cntnap2* KO mice. These findings suggest that changes in cellular and morphological organization of the MGN could potentially contribute to the atypical auditory processing phenotype in *Cntnap2* KO mice.

Finally, Chapter 7 provides a general discussion of the cumulative findings and general implications of the overall dataset in understanding the gene, brain, and behavior relationships that characterize the etiology of language-based neurodevelopmental disorders.

CHAPTER 2

A behavioral evaluation of sex differences in a mouse model of severe neuronal migration disorder.

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2.1 Abstract

Disruption of neuronal migration in humans is associated with a wide range of behavioral and cognitive outcomes including severe intellectual disability, language impairment, and social dysfunction. Furthermore, malformations of cortical development (MCD) have been observed in a number of neurodevelopmental disorders (e.g. autism and dyslexia), where boys are much more commonly diagnosed than girls (estimates around 4 to 1). The use of rodent models provides an excellent means to examine how sex may modulate behavioral outcomes in the presence of comparable abnormal neuroanatomical presentations. Initially characterized by Rosen et al. 2012, the BXD29-*Tlr4*$^{lps\text{-}2J}$/J mouse mutant exhibits a highly penetrant neuroanatomical phenotype that consists of bilateral midline subcortical nodular heterotopia with partial callosal agenesis. In the current study, we confirm our initial findings of a severe impairment in rapid auditory processing (RAP) in affected male mice. We also report that BXD29-*Tlr4*$^{lps\text{-}2J}$/J (mutant) female mice show no sparing of RAP, and in fact show deficits similar to mutant males. Interestingly, female BXD29-*Tlr4*$^{lps\text{-}2J}$/J mice *do* display superiority in Morris water maze performance as compared to wild type females, an affect not seen in mutant males. Finally, we report new evidence that BXD29-*Tlr4*$^{lps\text{-}2J}$/J mice, in general, show evidence of hyper-social behaviors. In closing, the use of the BXD29-*Tlr4*$^{lps\text{-}2J}$/J strain of mice—with its strong behavioral and neuroanatomical phenotype—may be highly useful in characterizing sex independent versus dependent mechanisms that interact with neural reorganization, as well as clinically relevant abnormal behavior resulting from aberrant neuronal migration.

2.2 Introduction

Disorders of neuronal migration show substantial heterogeneity in both neuroanatomical and behavioral outcomes, and have been linked to a wide range of neurological, neurobehavioral, and psychiatric disorders including epilepsy, schizophrenia, intellectual disability, autism, and dyslexia (Chang et al., 2005; Galaburda et al., 1985; Guerrini, 2005a; Wegiel et al., 2010; Yang et al., 2011). Associated neuropathology can include neural ectopias and dysplasias, polymicrogyria, and periventricular nodular heterotopia, all of which have been shown to be modulated by both genetic and/or environmental mechanisms (see Naumburg, Strömberg & Kieler, 2012 and Guerrini & Parrini, 2010 for review). These types of focal neuronal migration anomalies can lead to further disorganization of the developing cortex, including abnormal cortical layering and altered patterns of neuronal connectivity (Jenner, Galaburda & Sherman, 2000; Rosen, Burstein & Galaburda, 2000).

Given the range of neuropathologies associated with neuronal migration disorders, it is not surprising that the severity and types of behavioral and cognitive outcomes also vary greatly depending upon the location, degree, and extent of cortical disruption (see Guerrini & Parrini, 2010 for review). For example, in humans periventricular nodular heterotopia, polymicrogyria, and neural ectopias are generally seen to be localized to the perisylvian region—a well studied area implicated in language processing. Accordingly, these types of anomalies tend to be associated with language and reading disorders in humans (Boscariol et al., 2010a; Chang et al., 2005; Galaburda et al., 1985; Guerreiro et al., 2002). More diffuse neuronal migration anomalies such as classic lissencephaly and subcortical band heterotopia are more commonly associated

with severe intellectual disabilities and motor impairments (Barkovich et al., 1994; Dobyns et al., 1991; Guerrini, 2005a). However, there still remains a general multiplicity of behavioral, cognitive, and neurological outcomes that may result from a neuronal migration disorder, thus making overall patient outcomes difficult to clinically predict exclusively from an anatomical profile.

With regards to elucidating the relationship between different types of neuronal migration anomalies and behavioral outcomes, the use of rodent models has provided an invaluable tool. For example, rodent studies have examined disruption of normal cortical development through injury (focal freeze lesion; Clark et al., 2000c; Fitch et al., 1997; Fitch et al., 2008a; Threlkeld et al., 2006), embryonic exposure to teratogens (Threlkeld et al., 2009), and/or genetic factors (either intrinsic or genetically manipulated; Szalkowski et al., 2011; Szalkowski et al., 2012; Threlkeld et al., 2007). Results show that differing types of neuropathology can lead to different types of anomalous behavioral outcomes. For example, research examining rodent models of focally disrupted neuronal migration (similar to those observed in clinical populations with language and reading disorders) found different combinations of behavioral impairments associated with specific patterns of cortical disruption (Denenberg et al., 1991; Fitch et al., 2008a; Peiffer et al., 2001; Peiffer, Rosen & Fitch, 2002; Peiffer, Rosen & Fitch, 2004; Szalkowski et al., 2011; Szalkowski et al., 2012; Threlkeld et al., 2009; Threlkeld et al., 2006; Threlkeld et al., 2007; Threlkeld et al., 2012).

Within the clinical literature, additional evidence reveals that males are more commonly diagnosed with neurodevelopmental disorders, including those associated with language learning impairments, and are also at a greater behavioral disadvantage than

females when diagnosed with the same disorder (see Liederman, Kantrowitz & Flannery, 2005, for review). However, the mechanisms underlying these sex differences in incidence and severity of behavioral symptomatology remain unclear. Rodent models in the past have directly examined sex differences in both neuroanatomical and behavioral outcome of injury-induced and spontaneously occurring models of disrupted neuronal migration (Peiffer, Rosen & Fitch, 2002; Peiffer, Rosen & Fitch, 2004). These studies revealed sex differences in rapid auditory processing (RAP) ability—a behavior associated with and used to model fundamental aspects of language-related ability. Specifically, male subjects with cortical malformations were impaired in short duration auditory processing conditions in comparison to age-matched male controls (no cortical malformation), while female subjects with similar induced/spontaneous cortical malformations performed comparably to their age-matched female controls (Peiffer, Rosen & Fitch, 2002; Peiffer, Rosen & Fitch, 2004). Additionally, a study conducted by Rial et al. 2009, described impairments in short term social recognition memory in male mice with focally induced microgyria, while females with the same induced malformation showed no deficit (Rial et al., 2009). Together, these studies suggested that females with cortical malformations were not behaviorally impaired on acoustic RAP tasks, as well as a short term social recognition memory task, while comparable malformations in male subjects did lead to deleterious behavioral performance. Despite this experimental evidence, the clinical literature clearly includes reports of cognitive, language, and motor impairments in some females with neuronal migration anomalies—indicating that behavioral impairments can arise from migrational anomalies in females under *at least some circumstances* (Guerrini & Parrini, 2010).

In a study investigating the genetic modulation of neuronal migration in hundreds of strains of BXD recombinant inbred (RI) mice, it was found that a particular strain – BXD29/TyJ – showed bilateral midline nodular hetereotopia and partial callosal agenesis in mice born from 2004 onward. Those mice born prior to 1998 were unaffected (Rosen et al., 2013). Interestingly, earlier research, discovered that genetic variation had occurred in this same strain that made them insensitive to lipopolysaccharide, a bacterial endotoxin (Cook et al., 2004; Cook et al., 2006). Subsequent study revealed a repeated sequence added to both ends of the *Tlr4* gene. On the basis of these findings, BXD29/TyJ RI mice were re-derived from a 1979 cryopreserved embryonic stock and designated BXD29/Ty (wild type for *Tlr4*), while the RI strain with the *Tlr4* spontaneous mutation was redesignated as BXD29-*Tlr4*$^{lps\text{-}2J}$/J. Rosen and colleagues reported that the malformation observed in BXD29-*Tlr4*$^{lps\text{-}2J}$/J mice was 100% penetrant in both males and females, and the location of neuroanatomical anomaly was invariant (between retrosplenial cortex and somatosensory/visual cortices). Moreover, the malformation was found to develop in later stages of cortical migration (Rosen et al., 2013). In addition, specific and severe impairment in RAP was found in male mice, but with no concurrent behavioral deficits in spatial or nonspatial maze learning (Morris and nonspatial water maze, respectively), or sensorimotor ability (rotarod; Rosen et al., 2013). Concurrent examination of the coisogenic BXD29/Ty (wild type) mouse strain, revealed no aberrant neuroanatomical or behavioral profile (Rosen et al., 2013). However, genetic backcross experiments indicated that the neuroanatomical phenotype was most likely not mediated by the *Tlr4* spontaneous mutation indentified by Cook et al. 2004. Instead, results suggested at least

two autosomal recessive genes contributed to the observed phenotype (Rosen et al., 2013).

The current study was performed to provide a more comprehensive examination of the relationship between severe neuroanatomical malformation and behaviors clinically associated with neuronal migration disorders, including language-related and social dysfunctions, by expanding the neurobehavioral profile of the BXD29-*Tlr4*$^{lps\text{-}2J}$/J recombinant inbred strain of mouse. Additionally, an investigation of potential sex differences was undertaken to determine whether BXD29-*Tlr4*$^{lps\text{-}2J}$/J females display the same pattern of behavioral outcomes as their male BXD29-*Tlr4*$^{lps\text{-}2J}$/J counterparts. Based on prior data, we hypothesized that affected females might show no deficits (Peiffer, Rosen & Fitch, 2002; Peiffer, Rosen & Fitch, 2004). In this regard, examination of sex differences could provide insight into whether some form of developmental or behavioral compensation or "protection" in female subjects is evident in this particular model of highly disrupted neuronal migration.

We report here that we replicated our previous findings of a severe RAP impairment in male BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant mice (Rosen et al., 2013). Interestingly, concurrent examination of RAP in female BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant mice revealed no mutation by sex interaction, meaning that female BXD29-*Tlr4*$^{lps\text{-}2J}$/J mice performed comparably to BXD29-*Tlr4*$^{lps\text{-}2J}$/J males and were equally impaired relative to control females. However, further examination of Morris water maze learning did reveal sex differences in performance, with female BXD29-*Tlr4*$^{lps\text{-}2J}$/J exhibiting *superior* Morris water maze ability compared to both male BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant mice and female coisogenic controls. Finally, novel examination of social behavior and vocalizations

revealed that both male and female BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant mice displayed deviant social behaviors in comparison to BXD29/Ty wild type controls (though vocalizations could only be measured in males since adult females rarely vocalize Maggio & Whitney, 1985) These anomalies were unexpectedly found in a *hyper*-social direction.

2.3 Methods and Materials

2.3.1 Ethics Statement

All procedures were conducted in compliance with the National Institutes of Health and approved by the University of Connecticut's Institutional Animal Care and Use Committee (IACUC; protocol A09–050M).

2.3.2 Selection of Behavioral Tasks

The selection of behavioral tasks was based on the intent to; 1) replicate and expand the original behavioral findings described in Rosen et al. 2013; 2) examine potential sex differences using these tasks; and 3) to employ new assessments of social behaviors. Tasks examined included auditory processing tasks, rotarod, Morris water maze, male social vocalization behavior, and a social preference task. Our aims included: 1) to provide a more comprehensive neurobehavioral profile of BXD29-*Tlr4*$^{lps\text{-}2J}$/J utilizing a behavioral battery reflective of the wide range of cognitive and social behaviors associated with neuronal migration disorders and; 2) to examine whether severe neuroanatomical anomalies could lead to sexually dimorphic behavioral outcomes.

2.3.3 Subjects

For the series of behavioral experiments, ten male and ten female BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant (JAX stock number 000029) and ten male and ten female BXD29/Ty wild type (JAX stock number 010981) were obtained from the Jackson Laboratory (Bar Harbor,

ME) at postnatal day 29–36. Note that in subsequent text, BXD29-*Tlr4*$^{lps\text{-}2J}$/J will be referred to as “mutant” and BXD29/Ty will be referred to as “wild type”. All subjects arrived together at the University of Connecticut, Department of Psychology, and were single-housed in standard lab cages (12 h/12 h light/dark cycle) with food and water available *ad lib*. Subjects were behaviorally examined together in adulthood beginning on postnatal day 129 (P129). All procedures were performed blind to subject strain

2.3.4 Auditory Processing

2.3.4.1 The Startle Reduction Paradigm

Assessment of RAP was conducted utilizing a modified pre-pulse inhibition paradigm (PPI; see Fitch et al., 2008b, for detailed review). Briefly, the startle reduction paradigm exploits the subjects’ acoustic startle reflex (ASR)—a large amplitude motor reflex which is evoked by an unexpected, intense, auditory stimulus (startle eliciting stimulus; SES). However, a reduction in the ASR can be elicited with the presentation of a non-startling but salient, stimulus (i.e. pre-pulse or cue) 20–500 ms prior to the SES. If the subject is capable of detecting the auditory pre-pulse preceding the SES, then attenuation of the ASR is typically seen. In this way the PPI paradigm provides a means to examine auditory detection and discrimination using varied manipulations of the pre-pulse cue. Attenuation of the ASR to the SES is quantified and examined using an “attenuated score” (ATT), which is a percent comparison of the ASR amplitude over cued and uncued trials (cued ASR/uncued ASR*100).

During auditory testing, subjects were placed on individual load-cell platforms (MED Associates, St. Albans, VT). Voltage output from each load cell platform was sent through a linear amplifier (PHM-250-60 MED) into a Biopac MP100WS Acquisition

system (Biopac Systems, Goleta, CA). The MP100WS was connected to a Macintosh computer running Acqknowledge v 3.9.2, which recorded the ASR of the subject (in volts) for each trial following the presentation of a SES. For data analysis, the maximum peak value of the ASR was extracted from the 200 ms epoch following the onset of the SES. The magnitudes of peak values were coded for each cued and uncued trial (representing the subject's absolute response amplitude for each trial). Auditory stimuli were generated using a Dell Pentium IV PC with custom programs executed using the program RPvdsEx and a Tucker Davis Technologies (Alachua, FL) real time processor (RP2). Sounds were amplified using a Niles SI-1260 Systems Integration Amplifier (Niles Audio Corporation, Carlsbad, CA), and delivered via powered speakers located approximately 50 cm above each platform. For all auditory processing paradigms, the SES was a 50 ms broadband white noise "burst" presented at 105 dB.

2.3.4.2 Normal Single Tone

Prior to RAP examination, all subjects were assessed on the normal single tone task—a measure of baseline auditory and PPI ability. Specifically, the auditory PPI control task examined whether subjects exhibited hearing deficits and/or impaired gross motor reflexes which could confound further auditory PPI testing. Testing sessions consisted of 104 pseudorandomly presented cued and uncued trials at inter-trial intervals (ITI) of varying durations (16–24 s). The task comprised a silent background with the intermittent presentation of an intense SES (50 ms, 105 dB white noise burst) during uncued trials. However, cued trials were characterized by a salient, yet moderately intense, auditory cue (50 ms, 75 dB, 5000 Hz tone pip) presented 100 ms prior to the SES. A percent

comparison of cued and uncued response amplitudes (ASR) using an attenuated score (cued ASR/uncued ASR*100; ATT) were utilized for analysis.

2.3.4.3 Silent Gap Detection

The silent gap detection task examined the subject's ability to discriminate breaks (silent gaps) in continuous broadband white noise. Daily test sessions comprised 300 pseudorandomly presented cued and uncued trials, which were characterized by a continuous presentation of white noise (75 dB) with the occurrence of the SES at varying ITIs (16–24 s). Uncued trials consisted of a 0 ms gap condition (no cue) prior to the presentation of the SES, and provided the subjects' baseline ASR amplitude score relative to cued trials. During cued trials, silent gaps of variable duration were embedded within the background white noise and presented 100 ms prior to SES onset. Two different variations of the silent gap detection paradigm were utilized—a long duration gap and a short duration gap task. The long gap detection task utilizes silent gap durations ranging from 50 to 300 ms (SG 0–300 ms) across three consecutive days of testing, beginning on P132. For the short gap detection task, subjects were assessed using silent gap durations ranging between 2 to 100 ms (SG 0–100 ms) across four consecutive days of testing (beginning on P182). Again, attenuated scores (ATT) derived by a percent comparison of cued and uncued response amplitudes (cued ASR/uncued ASR*100) were assessed.

2.3.5 Sensorimotor Assessment: Rotarod

The rotarod task was used to examine subjects' general sensorimotor abilities and balance. On P156, subjects were individually placed on a rotating cylindrical drum that gradually accelerated from 4 to 40 rotations per minute over a span of two minutes.

Subjects were provided four consecutive test trials on the rotarod, and the length of time to remain on the rotating drum was recorded. Latency on the accelerating drum was averaged across the four trials for further analysis.

2.3.7 Water Maze Assessment

2.3.7.1 Visual Platform

All subjects were examined on the visual platform control task to rule out any potential underlying differences and/or impairments in motivation, swimming, or visual ability that could impair subjects' ability to effectively perform subsequent tasks (and thus exclude them from further water maze assessment). On P157, subjects were placed in one end of an oval tub (103 cm × 55.5 cm) filled with room temperature water. Here, they were required to swim to a visible platform (8.5 cm in diameter; 1 cm above water surface), located at the opposite end of the tub. Latency to reach the platform was recorded for analysis.

2.3.7.2 Morris Water Maze

The Morris water maze is a behavioral task that is commonly used to assess spatial learning and memory, and specifically the ability to locate the position of a submerged escape platform using various static extra maze cues. Beginning on P160, subjects were tested on the Morris water maze over a span of four consecutive test days (sessions). During each test session, subjects were given four trials to locate the submerged platform. However, for each test trial, the subject release point into the maze was selected pseudorandomly at one of the four compass locations around the maze (i.e. north, south, east, and west), with each point used once per test session. Subjects were allowed 45 seconds to complete the trial and find the escape platform. If the platform was not located

prior to the 45 second allotment, subjects were gently guided to the goal before removal from the maze. The position of the hidden platform remained static throughout all four test sessions. Latency to the escape platform was measured and recorded using a Sony camera integrated with a SMART video tracking program (Panlab, Barcelona, Spain).

2.3.8 Social Context Assessment

2.3.8.1 Male Vocalizations

Male mice produce ultrasonic vocalizations when they are in the presence of a female (and particularly during estrus), or can detect a female's urinary estrus pheromones (Sipos, Kerchner & Nyby, 1992). Unfortunately, female mice do not vocalize substantially (primarily at low levels to other females), and therefore we did not include females for this task (Maggio & Whitney, 1985). Specifically, we measured the vocalization emission of ten male BXD29/Ty and ten male BXD29-*Tlr4*$^{lps\text{-}2J}$/J mice when exposed to accumulated seven-day dirty bedding obtained from mature, age-matched, female BXD29/Ty and BXD29-*Tlr4*$^{lps\text{-}2J}$/J mice. Bedding from seven-days was used to ensure inclusion of estrus phase (which is a 4 day cycle; Nelson et al., 1982). On P251, male subjects were individually placed in a standard laboratory cage filled with the bedding, and here vocalization behavior was recorded for 120 seconds using a ¼ inch condenser microphone (Brüel & Kjær type 4136, Nærum, Denmark) suspended 10 cm above the test subject. The microphone signal was preamplified with a Brüel & Kjær type 2619 preamplifier and then amplified using a Brüel & Kjær type 2636 amplifier (Brüel & Kjær, Nærum, Denmark). The signal was digitized at a sampling rate of 200 kHz using a Tucker Davis Technologies (Alachua, FL) multifunction processor (RX6) and saved as a .wav file using a custom MATLAB program (MathWorks, Natick, MA) on a Dell

Pentium IV PC. Recorded sound waveforms were visualized and examined using Adobe Audition (Adobe, San Jose, CA). Total time spent vocalizing was calculated by extracting vocalization intervals (continuous vocalization epochs < 200 ms apart) from periods of silence (no vocalization behavior). Percent time vocalizing for each subject was calculated by dividing the duration of active vocalization by the total time (120 seconds), multiplied by 100.

2.3.8.2 Social Preference Task

The social preference task was adapted from a social approach task utilized by Nadler et al., (2004), and further detailed by Crawley, (2004), to examine social interaction behaviors of mice when presented with a stranger mouse (Nadler et al., 2004). This task was included because in the clinical literature for patients with malformations of cortical development (MCD) such as neuronal migration anomalies, these anomalies are also associated with aberrant social behaviors (Badaruddin et al., 2007; Fishman et al., 2011; Gomes et al., 2012; Jeret et al., 1987; Wegiel et al., 2010). The purpose of the task was to determine whether mutants displayed differential social preference (sociability) behaviors in comparison to wild types when presented simultaneously with a stranger mouse and a novel toy, as well as to examine potential sex differences in social preference associated with neuronal migration anomalies. The social preference apparatus was similar in design to those previously described (Crawley, 2004; Nadler et al., 2004). Briefly, the test box consisted of a 40.5 cm (length) × 21.5 cm (width) × 19 cm (deep) acrylic box separated into three chambers using two removable partitions. The removable partitions each contained openings that could open and close at the beginning and end of the test session to allow the mouse to freely move between the three chambers. The task was conducted

in a darkened room with the lamp situated ~20 cm above the center chamber. Prior to the initiation of the test trial, subjects were placed in the center chamber for two minutes with the doors of the partitions closed to prevent the subject from moving into the other chambers. This allowed the mouse to habituate to the center chamber. Before the beginning of the test trial, an age and sex-matched wild type stranger mouse was placed inside a 9 cm × 9 cm × 9 cm wire cage in one of the flanking chambers, while a novel object (toy rubber band ball) was placed inside a similar wire cage in the opposite flanking chamber. At the end of the habituation trial, the test trial began with the simultaneous opening of the partition doors to allow free movement among all three chambers. Using a stop watch, an observer (blind to strain) recorded the time intervals spent in each chamber over a span of five minutes. Following the end of the five minutes, the test subject was removed from the test chambers and returned to the home cage. Total time spent in each chamber (stranger mouse, center, and novel object) was measured in seconds, and the percent time the test subject spent with the stranger mouse over the novel object was calculated by subtracting the total time spent with the novel toy from the total time spent with the stranger mouse, divided by the total test session (300 sec), multiplied by 100 [{total time with stranger mouse (sec)—total with novel toy (sec)}/300(sec)]*100.

2.3.9 Histology

Following behavioral testing, all subjects were weighed, deeply anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (15 mg/kg), and transcardially perfused using a 0.9% saline solution followed by 4% paraformaldehyde. Brains were extracted, bottled in paraformaldehyde, and shipped to GDR at Beth Israel Deaconess Medical

Center for further histological preparation. Brains were embedded in 12% celloidin and sliced in the coronal plane at a 30 µm thickness. A 1-in-5 series of sections were mounted and stained for Nissl substance using Thionin. Stereo Investigator System (MBF Biosciences, Williston, VT, USA) integrated with a Zeiss Axio Imager A2 microscope (Carl Zeiss, Thornwood, NY) was utilized to confirm and analyze the presence of malformations in BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutants, as well as lack of malformations in BXD29/Ty wild types. Estimation of cortical and heterotopia volumes were performed using point counting and Cavalieri's estimator. For statistical analysis, heterotopia volumes were analyzed as percent of neocortical volume (total heterotopia volume/total neocortical volume × 100). Estimation of the number of neurons present in the heterotopia was assessed using the Optical Fractionator probe. A one-way ANOVA was used to determine the presence of Sex differences in BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant mice.

2.4 Results

2.4.1 Histological confirmation of neuromorphological phenotype—no sex differences in stereological analysis

Postmortem histological analysis using light microscopy revealed and confirmed the presence of bilateral midline subcortical nodular heterotopia in all (male and female) BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant subjects, as previously reported (Rosen et al., 2013; Figure 2.1.A). Visualization of male and female BXD29/Ty wild type brains did not reveal any gross abnormal neuromophological phenotype, as described elsewhere (Rosen et al., 2013; Figure 2.1.B). Analysis of variance revealed no significant Sex differences in heterotopia volume as a percentage of total neocortical volume *[F(1,18)=1.77, N.S.]* in mutant subjects. Mean volume of heterotopia was 2.42% (SEM±0.23) of neocortical

volume in female mutant subjects and 2.08% (SEM±0.11) in male mutant subjects. In addition, no Sex differences were observed in the estimated number of neurons present in the heterotopia *[F(1,18)=2.87, N.S.]*. Heterotopias contained an estimated 187,275 (SEM±12,014) neurons in female mutant subjects and 150,444 (SEM±18,132) neurons in male mutant subjects.

2.4.2 Male and female BXD29-Tlr4^{lps-2J}/J mutants show deficits in rapid auditory processing

2.4.2.1 Normal Single Tone

Results from the normal single tone auditory pre-pulse inhibition control task showed that BXD29/Ty males and females, as well as BXD29-*Tlr4*$^{lps-2J}$/J males and females, were all able to detect this simple cue as measured by a comparison of cued versus uncued ASR ($t(9) = 3.8$, $P<0.01$; $t(9) = 2.8$, $P<0.05$; $t(9) = 4.3$, $P<0.05$, $t(9) = 3.0$, $P<0.05$; respectively, Figure 2.2.A). These results provide evidence that both mutant and wild type subjects were equally able to hear the auditory cue and had normal pre-pulse inhibition, an interpretation confirmed by a lack of significant main effects of Strain *[F(1,36)=1.2, NS]*, Sex *[F(1,36)=1.6, NS]*, or Stain × Sex interaction *[F(1,36) < 1, NS]* for the attenuated (ATT) scores.

2.4.2.2 Silent Gap 0-300 ms

Analysis of silent gap ATT scores using a repeated measures ANOVA with between variables of Strain (2 levels: mutant and wild type) and Sex (2 levels: male and female), and within variables Day (3 levels) and Gap (9 levels), revealed a significant main effect of Strain *[F(1,36)=46.4, P<0.001]*, but no significant main effect of Sex *[F(1,36) < 1, NS]*, nor a Sex × Strain interaction *[F(1,36) < 1, NS]* (Figure 2.2.B). Overall, the results

from the silent gap 0–300 ms task show that mutant mice, regardless of sex, performed much worse than wild type mice. Furthermore, the lack of an interaction between Sex and Strain demonstrates that behavioral effects associated with the mutation are comparable across the sexes. Additionally, a significant interaction between Strain × Gap *[F(8,288)=10.3, P<0.001]* showed that wild type mice performed much better on the longer (easier) gap intervals (>125 ms) in comparison to mutant mice, while both groups had more difficulty detecting the shorter gap intervals (<125 ms). An examination of performance at individual gaps using a paired samples t-test comparing mean cued and uncued startle responses in the mutant mice found that they showed significant discrimination for silent gap intervals greater than 75 ms (thus confirming that differences in silent gap 0–300 ms performance between mutant and wild type mice were a result of poorer RAP ability in mutant mice and *not* an across-the-board failure of gap detection in the mutants). A similar gap detection analysis in wild type mice found that they were able to discriminate the cue at all gap durations down to 50 ms (as compared to the 75 ms mutant threshold; Figure 2.2.B).

2.4.2.3 Silent Gap 0–100 ms

A repeated measures ANOVA on ATT scores including Strain (2 levels: mutant and wild type) and Sex (2 levels: male and female) as between variables, and Day (4 levels) and Gap (9 levels) as within variables, revealed a significant main effect of Strain *[F(1,36)=64.9, P<0.001]*. However, there was no main effect of Sex *[F(1,36) < 1, NS]* or Sex × Stain interaction *[F(1,36) < 1, NS]* (Figure 2.2.C). As with silent gap 0–300 ms, results from the silent gap 0–100 ms task reflect an overall impairment of RAP in mutant mice in comparison to wild type mice, an effect seen regardless of sex. A significant

Strain × Gap interaction was also found *[F(8,288)=25.4, P<0.001]*, again indicating that wild type mice performed better than mutants particularly on the "easier" gap intervals (>50 ms), while both groups had similar difficulties in detecting the shorter gap intervals (<50 ms), thus reducing group differences due to task difficulty. Comparison of mean cued and uncued startle amplitude within the mutant group was again performed to assess silent gap detection at individual gaps. T-tests revealed that mutant mice were capable of cue discrimination at the 75 ms interval only, indicating that mutant mice were generally impaired on this more difficult task, but were still able to perform the task on an easier (longer) condition. Analysis of individual gap detection in wild type mice found that they were capable of performing silent gap 0–100 ms at all gap intervals down to 2 ms (Figure 2.2.C).

2.4.3 BXD29-Tlr4$^{lps\text{-}2J}$/J mutants show no impairment of sensorimotor ability

A univariate ANOVA of rotarod latencies including the between-subjects variables Strain (2 levels: mutant and wild type) and Sex (2 levels: male and female), was used to assess sensorimotor ability. This analysis revealed no significant differences across Strain *[F(1,36)< 1, NS]* (Figure 2.3). However, there was a significant main effect of Sex *[F(1,36)=6.8, P<0.05]*, showing that females remained on the rotarod significantly longer than males, regardless of strain. This observation has been reported elsewhere and is consistent with previous findings (McFadyen et al., 2003). The absence of a significant interaction between the variables Strain and Sex *[F(1,36)<1, NS]*, coupled with independent samples t-tests, confirmed that there were no strain differences in rotarod ability for either sex.

2.4.4 Female BXD29-Tlr4$^{lps\text{-}2J}$/J mutants show superior Morris water maze ability

2.4.4.1 Visible Platform

Initial assessment of water maze escape latencies using a visual platform control task found no main effects of Strain *[F(1,36)<1, NS]*, Sex *[F(1,36)=2.5, NS]*, or Strain × Sex interaction *[F(1,36)=2.6, NS]*, providing evidence that all subjects performed comparably in visually locating and swimming to the visible platform. Thus, all subjects were included for further Morris water maze testing.

2.4.4.2 Morris Water Maze (MWM)

Statistical analysis of MWM escape latencies across four trials using a repeated measures ANOVA with between subject variables of Strain (2 levels: mutant and wild type) and Sex (2 levels: male and female) and the within subject variable of Day (4 levels), did not reveal an overall effect of Strain *[F(1,36)<1, NS]* (Figure 2.4.A). However, there was a main effect of Sex *[F(1,36)=4.3, P<0.05]*, with females overall taking *less* time to locate the hidden platform. This result runs counter to typical reports wherein female rodents consistently perform worse than males on MWM and related spatial tasks (Cimadevilla et al., 1999; Guo et al., 2011; Roof, 1993). This unusual result may be explained by a significant Strain × Sex interaction *[F(1,36)=5.1, P<0.05]*, indicating the sex effect was driven by mutant females (Figure 2.4.B). In fact, further *post hoc* analysis using Fisher's least significant difference test found statistically superior performance of the female mutant mice on the Morris water maze as compared to all other groups. This effect was seen when compared not only to the male mutant mice, but also to the wild type males and females (*P<0.05*). Additional analysis also found a main effect of Day *[F(1,36)=7.16, P<0.001]*, indicating that all subjects improved on the task as testing progressed.

2.4.5 BXD29-Tlr4$^{lps\text{-}2J}$/J mutant mice exhibit enhanced social behaviors

2.4.5.1 Male Vocalizations

Examination of male vocalization to estrous female bedding using an independent samples t-test revealed a significant main effect of Strain *[t(18)=-4.07, P<0.001]*, indicating that male mutant mice spent significantly *more* time vocalizing when exposed to female bedding in comparison to male wild type mice (41% vs. 4%; Figure 2.5.A).

2.4.5.2 Social Preference Task

Analysis of social preference data (percent time with stranger mouse) using a univariate ANOVA with between subject variables Strain (2 levels: mutant and wild type) and Sex (2 levels: male and female) suggests a trend toward a significant main effect of Strain *[F(1,34)=2.6, P=0.1]* (Figure 2.5.B). Although no definitive conclusions can be drawn from this result due to a high level of variability, the trend shows that mutant mice spent a greater percent of time with the stranger mouse than the novel toy in comparison to wild type mice. In fact, these findings strongly suggest *increased* sociability in the mutant mice based on the fact that social preference scores were more than doubled in mutant mice. Finally, there was no main effect of Sex *[F(1,34)<1, NS]* or Strain × Sex interaction *[F(1,34)<1, NS]*, indicating that this enhanced social behavior was seen for both male and female mutants.

2.5 Discussion

Neuronal migration disorders clinically show a wide range of neuroanatomical and behavioral manifestations, with deficits ranging from mild to severe. Moreover, behavioral anomalies are seen across cognitive, social, and neurophysiological domains. Although the pattern and severity of behavioral and cognitive outcomes seem to vary

depending on extent and localization of the malformation(s), a precise diagnostic prediction of symptoms associated with specific anomalies has not been possible, and this issue requires additional study (see Guerrini & Parrini, 2010, for review). Furthermore, clinical data show a greater prevalence of males diagnosed with neurodevelopmental disorders associated with neuroanatomical anomalies (such as autism and developmental dyslexia), and males also tend to present with more severe behavioral outcomes related to language and reading disability (see Liederman, Kantrowitz & Flannery, 2005, for review). To better examine the relationship between severe neuroanatomical malformations, as well as the potential role of sex in the manifestation of behavioral abnormalities associated with language-related dysfunction and social context, male and female BXD29-*Tlr4*$^{lps\text{-}2J}$/J mouse mutants (and their coisogenic and sex matched controls) were examined using a battery of assessments intended to model core non-verbal aspects of language related dysfunction, sensorimotor, and social ability.

2.5.1 Deficits in rapid auditory processing behavior in BXD29-Tlr4$^{lps\text{-}2J}$/J mice, but no sex differences

Within the current study, we were able to replicate and expand upon the behavioral findings initially described in Rosen et al. 2013. Specifically, the BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant mouse strain again displayed severe impairments in RAP despite typical hearing and PPI ability. Although the wild type BXD29/Ty mice were capable of gap detection up to 2 ms in duration, mutant mice were only able to discriminate silent gaps longer than 75 ms in duration. In addition, despite significant gap detection at the longer intervals, the mutant mice were unable to attain the same level of discrimination as wild type mice even at longer durations.

Interestingly, both male and female mutant mice were comparably impaired in silent gap detection in comparison to their respective coisogenic controls, indicating no sex difference in RAP ability. This result deviates from findings associated with previous research where sex differences in RAP ability favoring females *were* seen in other rodent models of neuroanatomical malformation and cortical injury (Fitch et al., 1997; Herman et al., 1997; Hill, Threlkeld & Fitch, 2011; Peiffer, Rosen & Fitch, 2004). However, it must be noted that these studies which reported a female "advantage" in outcomes following disruptions in cortical development all utilized models of **injury** induced damage and disruption (Hill, Threlkeld & Fitch, 2011; Peiffer, Rosen & Fitch, 2004). Although the mechanisms of altered cortical development and subsequent reorganization leading to RAP impairment remain unclear, studies of injury-based models examining disrupted cortical development have also shown sex-dependent mechanisms associated with apoptosis, as well as data implicating a role for estrogen modulation in neuroprotective pathways (Hill & Fitch, 2012; McCullough & Hurn, 2003). Furthermore, subsequent research following the discovery of sex differences in outcomes using the induced focal microgyria model (Fitch et al., 1997) found that the presence of circulating androgens in males and females provided exogenously following injury resulted in behavioral deficits in treated females (Hill, Threlkeld & Fitch, 2011) and also aberrant reorganization of the medial geniculate nucleus (MGN; Rosen, Herman & Galaburda, 1999; Rosen, Herman & Galaburda, 1999). That is, females with induced microgyria (not exposed to androgens) displayed comparable MGN morphology to unaffected females, thus suggesting that sex differences observed in impaired RAP ability and thalamic anomalies could be explained in part by early androgen exposure concurrent to cortical

injury. Evidence from injury based models, including focal microgyria and neonatal hypoxia-ischemia models, thus suggest that sex-dependent mechanisms (which may in turn reflect modulating effects of androgens and/or estrogens) associated with apoptosis and cellular reorganization could contribute to the female advantage observed in subsequent RAP ability. In contrast to results from these injury models, cortical abnormality in the BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant in the current study was *not* a result of an experimentally induced injury that could initiate a sexually dimorphic apoptotic cascade. Therefore, the *lack* of sex differences in RAP ability in mutant mice suggests that gonadal steroids do *not* mediate mechanisms associated with severe RAP impairment when that altered behavior is genetically mediated. These results are consistent with a recent study conducted by Szalkowski et al. 2013, which examined behavioral sex differences following genetic knockdown (RNAi) of the rodent homolog for the dyslexia candidate risk gene, *DYX1C1* (*Dyx1c1* in rodents; Szalkowski et al., 2013). This study reported no sparing of female performance in RAP behavior, with *Dyx1c1* (RNAi) females showing deficits comparable to those seen in treated males. Taken together with evidence from injury based models, findings suggest that cortical disruption as a result of non-injury related genetic factors may not necessarily elicit a female advantage, even though such an advantage is often seen in outcomes following injury-induced developmental cortical disruption (Fitch et al., 1997; Hill, Threlkeld & Fitch, 2011; Peiffer, Rosen & Fitch, 2004). As a caveat, it must be noted that Peiffer et al. 2002, demonstrated a female advantage in RAP among a subset of male and female BXSB mice expressing Layer 1 ectopias. Findings from this study are difficult to interpret in the context of sex interactions with injury versus genetics, specifically because it is not

known what factors lead some BXSB mice and not others to express ectopias (Schrott et al., 1993).

2.5.2 Superior spatial water maze learning in female BXD29-Tlr4$^{lps\text{-}2J}$/J mutant mice

Superior Morris water maze performance observed in female BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant subjects was an unexpected finding in the current study, especially since prior studies with male mutant mice revealed comparable Morris water maze learning to male wild type mice. Moreover, this finding was intriguing because male rodents tend to perform better than females on the Morris water maze as a baseline (see D'Hooge & De Deyn, 2001, for review). However, there have been a few instances in the rodent literature reporting a female superiority in spatial learning ability using the Morris water maze (Schrott et al., 1993). For example, this sex-reversal was seen in BXSB mice, an autoimmune strain of mice known to have spontaneously occurring molecular layer neural ectopias in a subset of the population. In the study conducted by Schrott et al. 1993, female BXSB mice swam significantly faster to the hidden platform, while traveling a shorter distance to locate the escape platform. Although the results from Schrott et al. 1993 seem to correspond to the current findings associated with a female advantage in spatial learning in models of neuronal migration anomalies, it must be noted that the presence of neural ectopias in BXSB mice are not 100% penetrant in comparison to malformations observed in BXD29-*Tlr4*$^{lps\text{-}2J}$/J mice. Additionally, there was no analysis comparing ectopic versus non-ectopic female performance in this sample, thus it remains unclear whether the presence of a neuroanatomical anomaly in BXSB females could have contributed to better Morris water maze performance. Despite previous research alluding to a potential female advantage in spatial learning and memory ability

associated with neuronal migration anomalies, the novel finding of a female superiority associated with the Morris water maze task in BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant mice must be examined further to verify the results. An additional possibility is that enhancements are sometimes associated with deficits, and the Morris water maze "enhancement" may have only been evidenced in females due to their lower initial baseline performance.

2.5.3 Hyper-sociability in BXD29-Tlr4$^{lps\text{-}2J}$/J mutant mice

Within a social context, male mice may produce ultrasonic vocalizations when they encounter a female mouse (particularly in estrus), or are exposed to female urinary pheromones (Dizinno, Whitney & Nyby, 1978; Holy & Guo, 2005; Nyby et al., 1983; Sipos, Kerchner & Nyby, 1992). We found that when male BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant and BXD29/Ty wild type mice were exposed to dirty female bedding (bedding containing estrus female urine), mutant mice spent a substantially greater amount of time emitting ultrasonic vocalizations as compared to wild type mice (41% vs. 4% of session). Additionally, comparison of social preference to a stranger mouse versus a novel inanimate toy found a trend that suggested mutant mice, regardless of sex, preferred *more* social interaction with the stranger mouse in comparison to wild type mice (8% social preference in wild type, 25% preference in mutants). Together these two findings suggest that BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutants exhibit hyper-social behaviors in comparison to BXD29/Ty wild type mice.

Previous work examining normal male mouse ultrasonic vocalization behavior to female urine found that sexually naïve males (as investigated in the current paradigm) do produce vocalizations to concentrated amounts of freshly voided female urine (in the absence of a female). However, sexually experienced males (provided female interaction

three minutes a day for eight days) vocalized significantly more when exposed to the same stimulus (Sipos, Kerchner & Nyby, 1992). Furthermore, it was found that both sexually naïve and experienced males vocalized less when exposed to concentrations of older urine (1–13 hours), and that vocalization behavior decreased over time when exposed to female urine without the presence of a female mouse (Sipos, Kerchner & Nyby, 1992). These findings suggest that results could also reflect differences in "social acuity" regarding whether a fertile female may actually be present. Specifically, wild type male BXD29/Ty subjects exhibited minimal vocalization behaviors (<4% of total session), and this might be expected since female urine concentration was more diffuse within the dirty female cage bedding and most likely contained a higher concentration of older urine (>1 hour) in comparison to freshly voided. However, when exposed to identical female dirty bedding, mutant BXD29-*Tlr4*$^{lps\text{-}2J}$/J mice emitted ultrasonic vocalizations for more than 40% of the total test session. This indicated that mutant mice were overtly sensitive to the dirty bedding stimulus, which resulted in a production level of vocal social behaviors more typical of that seen when a female is either physically present, or thought to be nearby (Dizinno, Whitney & Nyby, 1978; Holy & Guo, 2005; Nyby et al., 1983; Sipos, Kerchner & Nyby, 1992). Thus, one interpretation could be that mutant males have impairments in processing pheromonal (social) information in terms of relevance. On the other hand, our assessments of social preference also suggest that mutant mice are more social than wild type and prefer social interaction with another mouse in comparison to a novel inanimate object at higher than normal levels. Specifically, mutant mice spent 25% more time with the same sex stranger mouse versus the toy in comparison to 8% more time observed in wild type mice. Therefore, data

collectively from the two paradigms assessing behavioral response to different social contexts suggest that mutant BXD29-*Tlr4*$^{lps\text{-}2J}$/J mice exhibit more hyper-social behaviors in comparison to BXD29/Ty wild type controls.

2.5.3 BXD29-Tlr4$^{lps\text{-}2J}$/J mutant mice: a neuroanatomical model for neurodevelopmental disorders?

There are numerous molecular pathways that regulate neuronal migration (see Liu, 2011, for review). Several of these gene networks—identified through association studies using different types of gene variants including single nucleotide polymorphisms and copy number variants – have been implicated in complex neurodevelopmental disorders ranging from autism, schizophrenia, and developmental dyslexia (Fisher & Francks, 2006; Gilman et al., 2012; Glessner et al., 2009). Unfortunately, the molecular etiology of the aberrant neuroanatomical phenotype observed in BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant mice remains unknown. However, previous genetic backcross experiments observing the pattern of inheritance for the cortical malformations do suggest that the phenotype is mediated by the expression of two independent autosomal recessive genes, but additional sequencing experiments comparing mutant and wild type mice must be conducted in order to further isolate and identify the responsible genes (Rosen et al., 2013). It must be noted that the *Tlr4* mutation identified by Cook and colleagues was determined to not be causal in the development of the cortical mutations (Cook et al., 2006; Rosen et al., 2013).

Despite the lack of clear molecular insights associated with the BXD29-*Tlr4*$^{lps\text{-}2J}$/J model, the complete neuroanatomical phenotype of BXD29-*Tlr4*$^{lps\text{-}2J}$/J mice, including partial callosal agenesis and subcortical nodular heterotopia bilaterally located near the

midline between the retrosplenial cortex and parietal and visual cortices do share overlapping (though not identical) characteristics of other malformation phenotypes commonly observed in human disorders of neuronal migration. For example, the subcortical location and rather diffuse nature of the malformation is similar in respects to subcortical band heterotopia (Bai et al., 2003; Guerrini & Parrini, 2010; Rosen et al., 2013). However, the nodular structure of the BXD29-*Tlr4*$^{lps\text{-}2J}$/J malformation is not analogous to subcortical band heterotopia, and is more reminiscent of periventricular nodular heterotopia—a malformation located along the ventricles (Guerrini & Parrini, 2010; Rosen et al., 2013). The presence of periventricular nodular heterotopia has been indentified in patients with both dyslexia and Williams syndrome (Chang et al., 2005; Ferland et al., 2006)—disorders that are associated with impairments in reading ability and excessive sociability, respectively (Fishman et al., 2011; Shaywitz & Shaywitz, 2005). Behaviors associated with both dyslexia *and* Williams syndrome have been modeled in the current study using RAP and sociability paradigms, respectively. Importantly, we found altered behavior associated with the BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant mice within both of those behavioral domains.

Similarly, callosal agenesis in the human population is often also observed with other MCD, and is associated with a wide range of aberrant behaviors across cognitive and social domains (Badaruddin et al., 2007; Jeret et al., 1987). For example, atypical callosal morphology featuring a shorter and thinner corpus callosum is present in a subset of patients with Williams syndrome (Luders et al., 2007). However, notably, our pattern of findings do not provide an ideal match for Williams syndrome, since this population presents with an atypical presentation of language (though reading impairments are in

fact associated with intellectual disability in this population Laing et al., 2001). Nonetheless, the current mouse model could provide a platform to trace associations between specific neural anomalies and functional anomalies relevant to a range of disorders.

2.6 Conclusion

We report novel findings of RAP deficits in both male and female mutant BXD29-*Tlr4*$^{lps\text{-}2J}$/J mice. We also present evidence for enhanced female BXD29-*Tlr4*$^{lps\text{-}2J}$/J performance in Morris water maze learning, as well as hyper-sociability in mutant mice, regardless of sex. In closing, the BXD29-*Tlr4*$^{lps\text{-}2J}$/J mouse is not a perfect model for a singular neurobehavioral disorder, nor does it fit within a single phenotype of known human neuronal migration disorders. However, various components of the behavioral and neuroanatomical phenotype of BXD29-*Tlr4*$^{lps\text{-}2J}$/J mice share commonalities that can be translated to different types of neuronal migration and neurobehavioral disorders, allowing for a general examination of the relationship between neuronal migration malformation and behavior. Moreover, the lack of sex differences observed in RAP behavior within the mutant group do not directly address the lower incidence ratios favoring females over males in the diagnosis of dyslexia, SLI, and autism (2:1, 3:1, and 4:1, Baron-Cohen et al., 2011; Liederman, Kantrowitz & Flannery, 2005; Whitehouse, 2010). As such, the question remains why more males are diagnosed with language related neurodevelopmental disorders, many of which have a strong genetic component. For example, it may be that other environmental risk factors that exacerbate or interact with genetic risk (i.e., perinatal fevers, teratogens) may be the locus of female "advantage" as seen in rodent models of developmental injury and cortical disruption

(Fitch et al., 1997; Hill, Threlkeld & Fitch, 2011; Peiffer, Rosen & Fitch, 2004). Questions regarding the associations between genetic and environmental risk, sex, anomalies in cortical development, and subsequent impairments in behavior and outcomes remain a critical ongoing issue in neurodevelopment research. Importantly, our findings suggest that the BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant model may provide an ideal platform for continuing to tease apart these issues in future studies. As such, additional research using the BXD29-*Tlr4*$^{lps\text{-}2J}$/J strain should be undertaken to examine the relationship between neuronal migration anomalies, neural connectivity, behavior, and the role of sex in the manifestation of clinical outcome. The insights provided by these future studies could provide crucial clues to develop a neuroanatomical and reorganizational neurocircuitry that may explain how aberrant neuronal migration insult can lead to impairments within specific behavioral domains.

Acknowledgements

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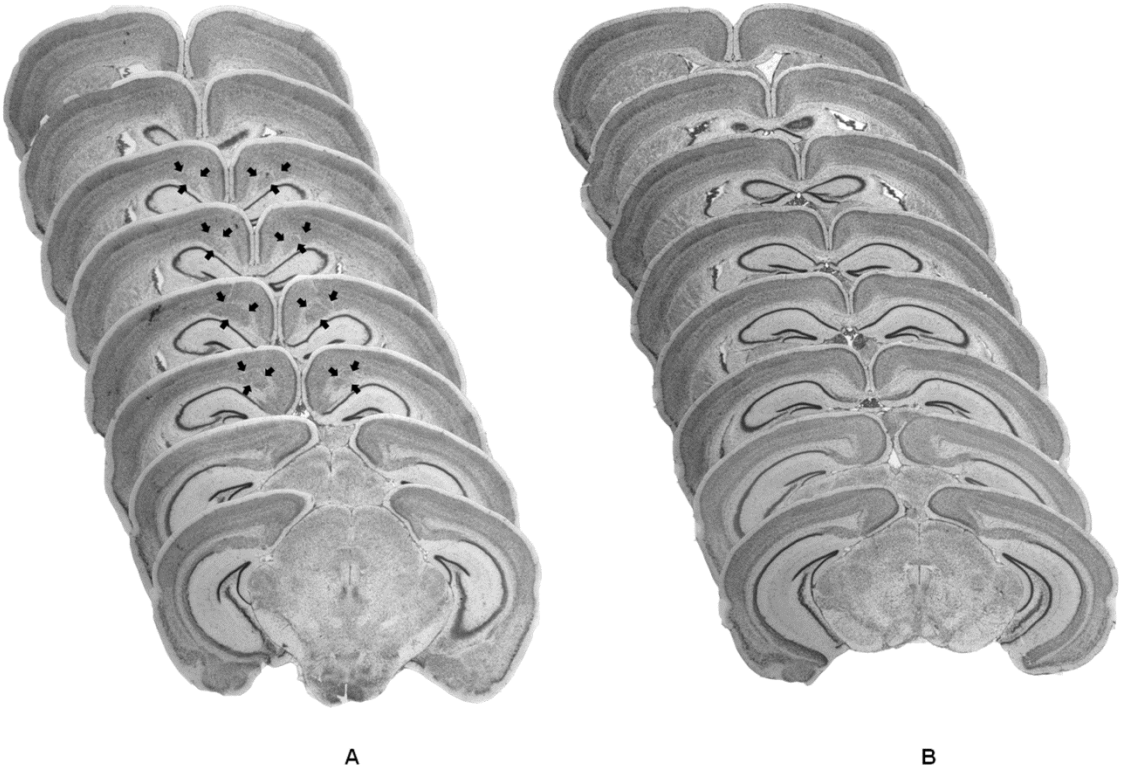

Figure 2.1. Histological examination of Nissl stained coronal sections in BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant and BXD29/Ty wild type mice. A) Bilateral midline subcortical nodular heterotopia are present in all BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant mice. Black arrows indicate the boundaries of the abnormal clustering of cells within the coronal plane. B) Visualization of both male and female BXD29/Ty wild type brains show no evidence of abnormal gross neuromorphology.

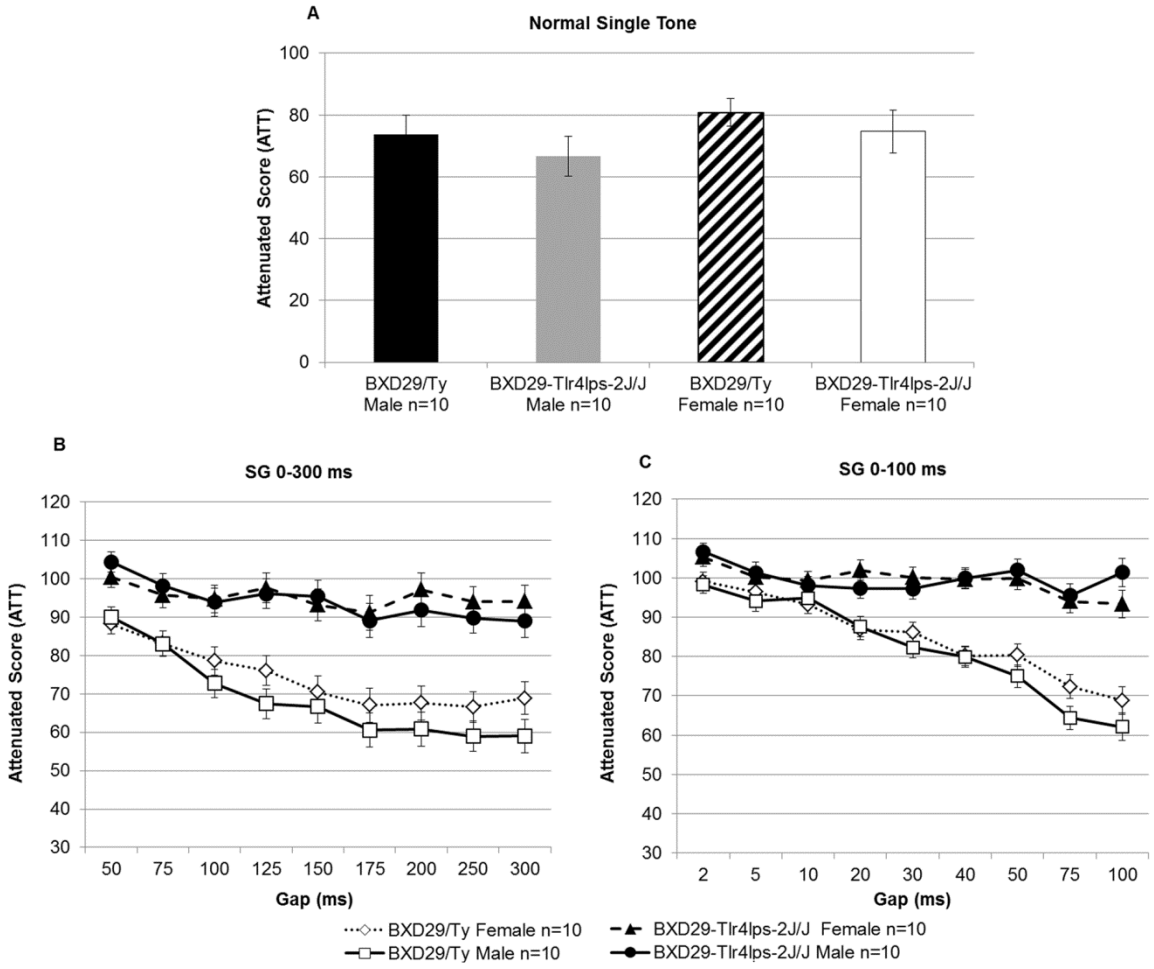

Figure 2.2. Auditory processing of BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant and BXD29/Ty wild type mice. A) Assessment of baseline auditory pre-pulse inhibition indicated comparable hearing and pre-pulse inhibition ability across Sex and Strain. Note, a lower attenuation score indicates better auditory pre-pulse inhibition ability. B) Silent Gap 0–300 ms and C) Silent Gap 0–100 ms gap detection tasks showed that BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant mice were specifically impaired in RAP. The lack of Sex × Strain interaction signified no sex differences in RAP across both tasks, suggesting that female BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant mice did not exhibit a behavioral sparring of RAP ability.

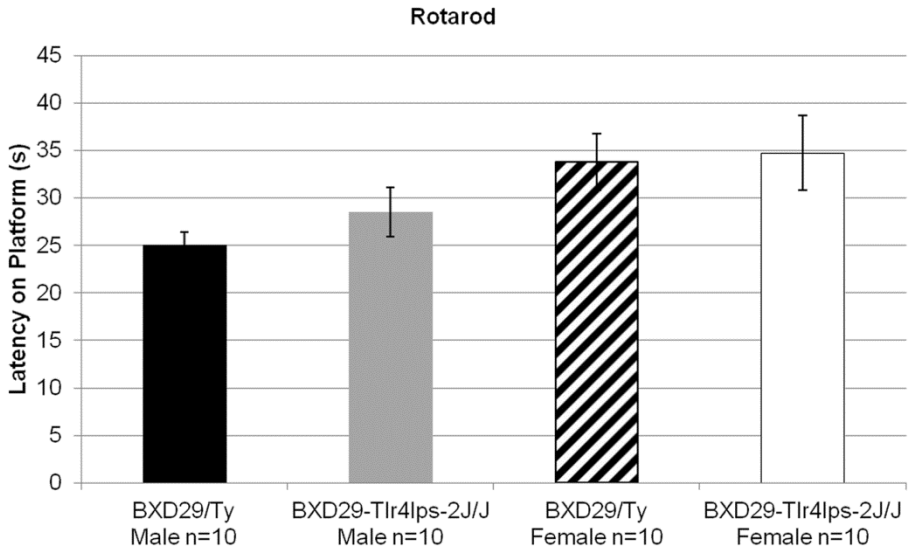

Figure 2.3. Rotarod performance of BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant and BXD29/Ty wild type mice. No main effect of Strain or Sex × Strain interaction was observed on the rotarod task, demonstrating the lack of sex differences in the BXD29-*Tlr4*$^{lps\text{-}2J}$/J strain of mice.

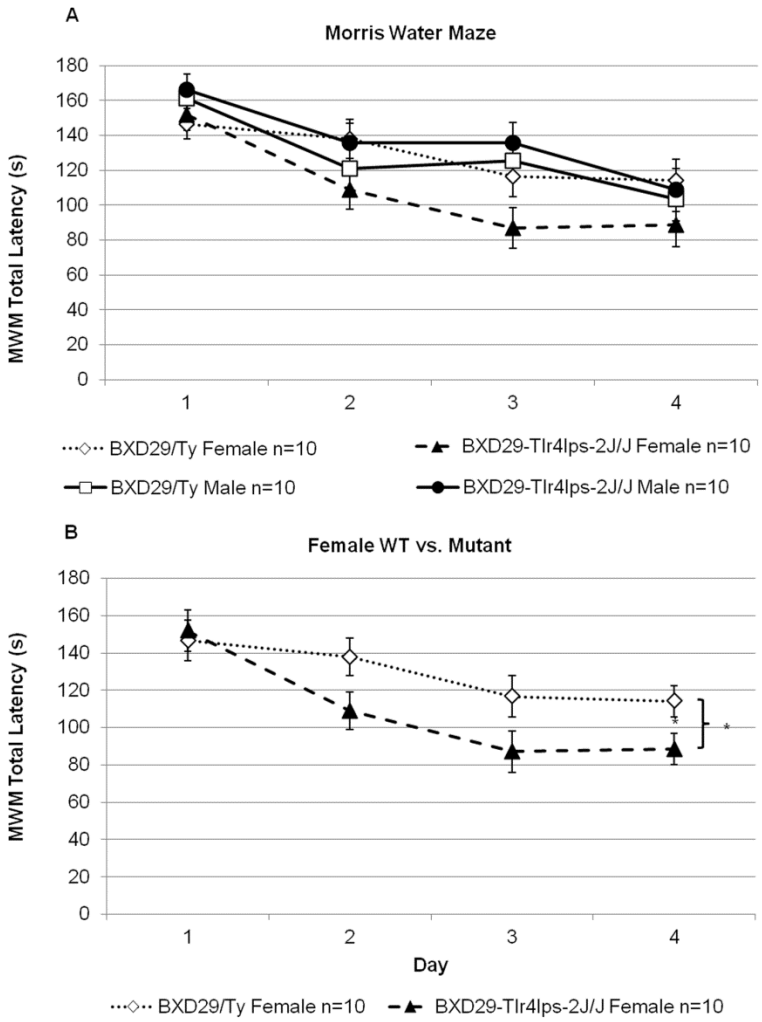

Figure 2.4. Morris water maze performance of BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant and BXD29/Ty wild type mice. A) Overall analysis of Morris water maze performance revealed a main effect of Sex and Sex × Strain interaction. B) Further *post hoc* analysis indicated that the main effects of Sex and Sex × Strain interaction were pulled by the significantly better Morris water maze performance of female BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant mice in comparison to both male BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant mice and female BXD29/Ty wild type mice. *P<0.05

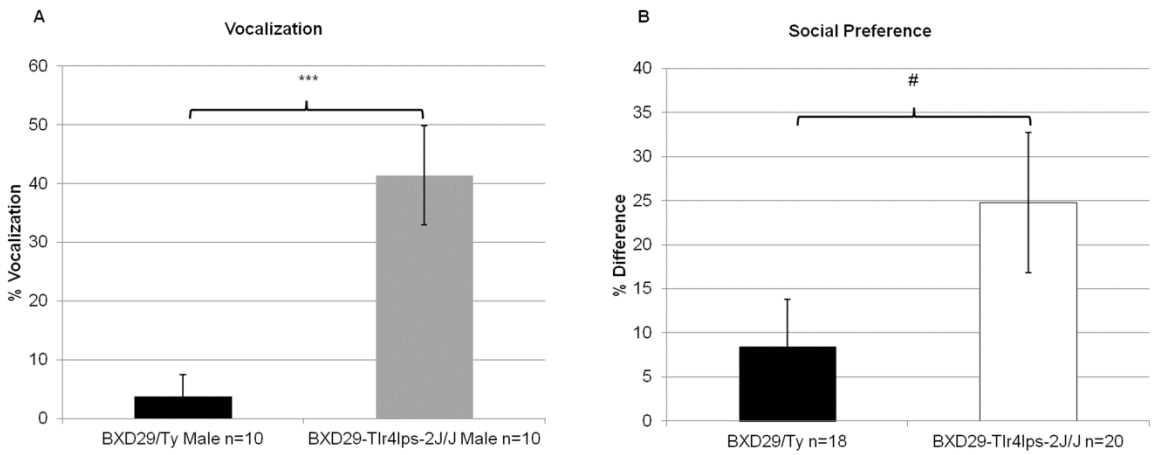

Figure 2.5. Examination of behavioral response to different social contexts in BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant and BXD29/Ty wild type mice. A) Analysis of male BXD29-*Tlr4*$^{lps\text{-}2J}$/J and BXD29/Ty ultrasonic vocalization emissions when exposed to seven-day-old dirty female bedding demonstrated that male BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant mice spent significantly more time vocalizing in response to dirty female bedding in comparison to male BXD29/Ty wild type mice. B) Social preference data revealed a nearly significant trend that suggested BXD29-*Tlr4*$^{lps\text{-}2J}$/J mutant mice, in general, may prefer increased social interaction with a stranger mouse in comparison to a novel inanimate object. #P=0.1; ***P<0.001

CHAPTER 3

Morphometric changes in subcortical structures of the central auditory pathway in mice with bilateral nodular heterotopia

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3.1 Abstract

Malformations of cortical development (MCD) have been linked to auditory processing impairments, as well as morphological changes in the auditory thalamic nucleus (medial geniculate nucleus; MGN) in human reading and language impaired populations, as well as in rodent models. Previous neuroanatomical examination of a BXD29 recombinant inbred strain (BXD29-*Tlr4*$^{lps-2J}$/J) revealed bilateral subcortical nodular heterotopia with partial callosal agenesis, and subsequent behavioral characterization showed a severe impairment in auditory processing on a silent gap detection task—an anomalous behavioral phenotype seen across both male and female BXD29-*Tlr4*$^{lps-2J}$/J mice. In the present study we expanded upon the neuroanatomical and behavioral findings in the BXD29-*Tlr4*$^{lps-2J}$/J mutant mouse by investigating whether subcortical changes in cellular morphology are present in neural structures critical to central auditory processing (including the MGN, and the ventral and dorsal subdivisions of the cochlear nucleus; VCN and DCN, respectively). Stereological assessment of brain tissue obtained from both male and female BXD29-*Tlr4*$^{lps-2J}$/J mice previously tested on a battery of auditory processing tasks revealed overall smaller neurons in the MGN of BXD29-*Tlr4*$^{lps-2J}$/J mutant mice in comparison to BXD29/Ty coisogenic controls, regardless of sex. Interestingly, examination of the VCN and DCN revealed sexually dimorphic changes in neuronal cell size, with a distribution shift toward larger neurons in female BXD29-*Tlr4*$^{lps-2J}$/J brains. These effects were not seen in males. Together, the combined data set supports and further expands the association between MCD, auditory processing impairments, and changes in subcortical anatomy of the central auditory pathway. The

current stereological findings also highlight sex differences in neuroanatomical presentation in the presence of a common auditory behavioral phenotype.

3.2 Introduction

Cortical neuronal migration is a critical step in the development of the brain, and lays the foundation for the formation of intricate thalamocortical, corticothalamic, and other connections crucial to the processing of complex information. This stage of brain development is highly regulated by numerous genetic and hormonal mechanisms (see Gleeson & Walsh, 2000 and McEwen, 1992, for review). Given the precise concerted organization of cortical formation, it is no surprise that disruptions in neocortical neuronal migration have been associated with a wide range of neurological, neurobehavioral, and cognitive dysfunction including epilepsy, schizophrenia, intellectual disability, autism spectrum disorder, and language and reading disability (Boscariol et al., 2010a; Boscariol et al., 2009; Chang et al., 2005; Galaburda et al., 1985; Guerrini, 2005b; Liu, 2011; Strauss et al., 2006; Wegiel et al., 2010; Yang et al., 2011). As a result of these malformations of cortical development (MCD), further disorganization of the developing cortex can occur, including abnormal cortical layering and changes in neural circuitry (Chevassus-au-Louis et al., 1999; Christodoulou et al., 2012; Christodoulou et al., 2013; Im et al., 2014; Jenner, Galaburda & Sherman, 2000; Rosen, Burstein & Galaburda, 2000; Threlkeld, Rosen & Fitch, 2007).

In a seminal study conducted by Galaburda et al. (1985) *post mortem* histological analysis of four male dyslexic brains revealed MCD that consisted of layer 1 neural ectopias, underlying cortical dysplasias, and micropolygyria located around the perisylvian region of the cortex—an area associated with language and reading. Years later, these findings were extended to *post mortem* histological analysis of three female dyslexic brains showing similar malformations, specifically molecular layer ectopias and

cortical dysplasia (although to a quantitatively lesser extent; Humphreys, Kaufmann & Galaburda, 1990). Subsequent *in vivo* studies have also reported varied MCD associated with language and reading impairment, including reductions in cortical gray matter (Brambati et al., 2004; Kronbichler et al., 2008; Menghini et al., 2008; Richlan, Kronbichler & Wimmer, 2013), atypical white matter organization and callosal morphology (Chang et al., 2007; Darki et al., 2012; Hasan et al., 2012; Klingberg et al., 2000; Niogi & McCandliss, 2006), periventricular nodular heterotopia (Chang et al., 2005), and polymicrogyria (Boscariol et al., 2010b). Furthermore, additional *post mortem* investigation of human dyslexic brains also indicated abnormal subcortical anatomy in visual nucleus of the thalamus (lateral geniculate nucleus, LGN) as well as the auditory nucleus of the thalamus (medial geniculate nucleus; MGN; Galaburda & Eidelberg, 1982; Galaburda, Menard & Rosen, 1994; Livingstone et al., 1991). Here, dyslexic brains showed changes exclusively in the magnocellular layers of the LGN, but not the parvocellular layers (Livingstone et al., 1991). Specifically, mean cell area was smaller in the magnocellular layers, a finding that corresponded to physiologic data showing that dyslexics had diminished evoked potentials to visual stimuli related to magnocellular (rapid, low contrast) processing, but not to parvocellular (slow, high-contrast) dependent visual processing (Livingstone & Hubel, 1988; Livingstone et al., 1991). Interestingly, a similar pattern of atypical neuronal morphology was also present in the MGN of dyslexics, with more small and fewer large neurons (Galaburda, Menard & Rosen, 1994). This finding is further supported by recent functional imaging data showing abnormal activity in the region of the MGN in dyslexic individuals during an auditory phoneme discrimination task (Díaz et al., 2012). Numerous studies also report impaired processing

of rapid, but not slow, auditory information in language and reading impaired populations, including SLI and dyslexia, and even at-risk infants (Benasich & Tallal, 2002; Boscariol et al., 2010b; Choudhury et al., 2007; Cohen-Mimran & Sapir, 2007; Hari & Renvall, 2001; Raschle et al., 2013; Tallal & Piercy, 1973; Tallal, 1980; Tallal & Newcombe, 1978; Vandermosten et al., 2011). Although it must be noted that a distinct magnocellular sub-system (present in the LGN) has not been identified in the MGN, the observation of smaller cells in both the LGN and MGN of dyslexics may point to a generalized neurobiological "signature" associated with poor sensory processing of rapidly changing information (see Stein, 2001, for review).

Rodent models of MCD further support a link between cortical malformations, anomalous subcortical anatomy, and rapid auditory processing (RAP) deficits. Male rats with injury-induced cerebrocortical microgyria (comparable in structure to those found in dyslexic individuals studied by Galaburda et al. (1985)) also showed impairments in RAP (Clark et al., 2000a; Clark et al., 2000c; Fitch et al., 1997). Subsequent examination of neuronal cell size in the MGN of male rats with injury-induced microgyria revealed not only smaller cells in general, but a shift in the distribution of cell sizes toward the left (smaller cells) compared to sham controls (Herman et al., 1997; Rosen et al., 2006; Rosen, Herman & Galaburda, 1999). Genetic manipulation of rodent homologs of candidate dyslexia risk genes such as *Dyx1c1* also have been shown to lead to MCD (e.g., neural ectopias in layer 1 of the cortex and white matter heterotopia; Rosen et al., 2007; Szalkowski et al., 2013; Threlkeld et al., 2007). Behaviorally, rats with a genetic knockdown of *Dyx1c1* (via RNA interference; RNAi) also showed RAP deficits, as well as a shift in cell size distribution of the MGN toward smaller neurons (Szalkowski et al.,

2013; Threlkeld et al., 2007). Interestingly, behavioral deficits observed in both injury-induced and genetic models of MCD were specific to short (rapid) duration auditory cues, and were not seen for long duration (slow) auditory stimulus processing.

Striking similarities in the neuroanatomical and behavioral patterns across neurodevelopmentally atypical clinical populations and rodent models together suggest that pathological changes in the developing brain could underlie low-level sensory processing deficits reported, contributing in turn to higher order impairments in humans. Furthermore, it is known that early disruption of cortical development is capable of reorganizing the brain and altering connectivity (Chevassus-au-Louis et al., 1999; Christodoulou et al., 2012; Christodoulou et al., 2013; Goldman & Galkin, 1978; Im et al., 2014; Jenner, Galaburda & Sherman, 2000; Rosen, Burstein & Galaburda, 2000). Therefore, it is likely that alterations in reciprocal cortico-thalamic/thalamo-cortical interactions, as well as structural dysgenesis in other central auditory pathway structures (e.g., MGN, inferior colliculous, cochlear nucleus), may also be present.

Previously we reported severe RAP impairments in male and female BXD29-*Tlr4*$^{lps-2J}$/J mice—a recombinant inbred strain of mice with highly penetrant (100%) MCD characterized by bilateral midline nodular heterotopia and partial callosal agenesis (Rosen et al., 2013; Truong et al., 2013). Based on prior literature detailing structural atypicalities in the MGN associated with difficulties in RAP in both clinical and rodent models (Galaburda, Menard & Rosen, 1994; Herman et al., 1997; Szalkowski et al., 2013), the aim of the current study was to determine not only whether a similar pattern of dysgenic MGN morphology would be observed in male and female BXD29-*Tlr4*$^{lps-2J}$/J mutant mice, but also whether these anomalies might extend to lower structures along the

central auditory pathway, including the cochlear nucleus (CN)—a structure that has direct functional connectivity to the MGN, and allows for fast relay of auditory information (Anderson et al., 2006; Schofield et al., 2014). Using stereological methods, we estimated cell numbers and measured cell sizes of neurons in the MGN, as well as the dorsal and ventral subdivisions of the CN in male and female BXD29-*Tlr4*$^{lps-2J}$/J mutant and matched coisogenic BXD29/Ty controls (the latter showing no evidence of MCD). Findings revealed smaller cells overall in the MGN of both BXD29-*Tlr4*$^{lps-2J}$/J male and female mutant mice as compared to BXD29/Ty mice. However, cell size analysis in the ventral cochlear nucleus (VCN) and dorsal cochlear nucleus (DCN) both showed a shift toward larger cells in BXD29-*Tlr4*$^{lps-2J}$/J female mice, but not in BXD29-*Tlr4*$^{lps-2J}$/J male or BXD29/Ty male and female subjects.

3.3 Materials and Methods

3.3.1 Subjects

Ten male and ten female BXD29-*Tlr4*$^{lps-2J}$/J mutant (JAX stock number 000029) and ten male and ten female BXD29/Ty wildtype (JAX stock number 010981) were obtained from the Jackson Laboratory (Bar Harbor, ME) at postnatal day (P) 29–36. In subsequent text, BXD29-*Tlr4*$^{lps-2J}$/J will be referred to as “mutant” and BXD29/Ty will be referred to as “WT” (wild type). Detailed descriptions of behavioral testing and results conducted on these subjects were reported elsewhere (Truong et al., 2013). Briefly, subjects were assessed on a variable duration silent gap task (0–300 ms and 0–100 ms) to examine RAP ability. Additional testing included measures from a rotarod task, Morris water maze, a social preference task, and male vocalization behaviors in a socially relevant context.

3.3.2 Histology

Following the end of behavioral testing (~ P265), subjects were weighed, anesthetized using a cocktail of ketamine (100 mg/kg) and xylazine (15 mg/kg) and transcardially perfused using a 0.9% saline solution followed by 4% paraformaldehyde. Brains were then removed from the skull, weighed, bottled, post fixed in 4% paraformaldehyde, and shipped to GDR at Beth Israel Deaconess Medical Center for further histological preparation. Brains were embedded in 12% celloidin and serially sectioned by microtome in the coronal plane at 30 µm (Leica SM2000 R, Leica Biosystems, Buffalo Grove, IL). Every fifth section was stained for nissl substance using thionin, mounted on glass slides, and coverslipped using permount. Right and left hemispheres were identified by a notch created above the left hemisphere following the celloidin embedding procedure.

3.3.3 Stereological measures

All prepared tissue samples were analyzed using Stereo Investigator (MBF Biosciences, Williston, VT, USA) integrated with a Zeiss Axio Imager A2 microscope (Carl Zeiss, Thornwood, NY). Experimenters were blind to subject strain and sex. Only neurons were assessed in the series of stereological experiments, and neurons were distinguished from non-neuronal cell types by the presence of a single distinct nucleolus within the cell nucleus. Neuronal cell populations were estimated using the optical fractionator probe, with cross sectional neuronal cell area estimated concurrently using the nucleator probe. Neurons were only counted for analysis if the nucleolus was in focus and within the appropriate boundaries of the active counting frame and dissector depth (see below). A

standard sterotaxic atlas was used to determine the borders of the MGN, VCN, and DCN for measurement purposes (Paxinos & Watson, 1986).

3.3.3.1 MGN cell measures

An average of 5 sections per brain contained the MGN and were used for analysis. Contours outlining the entirety of the MGN were drawn at 2.5X magnification. Cell size measurements and counts were assessed at 100X magnification. A sampling grid size of 150 µm × 150 µm and a 30 µm × 30 µm counting frame were used for stereological examination. Estimates for both neuronal cell population and neuronal cell area were obtained for left MGN, right MGN, and total (left + right) MGN for each subject.

3.3.3.2 VCN and DCN cell measures

An average of 7 and 5 sections per brain contained the VCN and DCN (respectively), and these were used for analysis. Contours outlining the VCN and DCN (separately) were drawn at 2.5X magnification, with cell size measurements and population estimates obtained at 100X magnification. A sampling grid size of 150 µm × 150 µm, and a 30 µm × 30 µm counting frame, were used for stereological analysis. Estimates for both neuronal cell population and neuronal cell area were obtained for left, right, and total (left + right) VCN and left, right, and total (left + right) DCN.

3.3.4 Statistical Analysis

Mean cell count and mean cell size for MGN, VCN, and DCN were analyzed using a univariate ANOVA. Cell size distribution analysis was conducted using a cumulative percent distribution. To examine group differences in cell size distribution a

nonparametric analysis (Kruskal-Wallis) was used to assess overall differences across group medians. Follow-up *post hoc* analysis of pairwise comparisons within Sex was also conducted using the Kruskal-Wallis test, with follow-up analysis using a 2-independent sample Kolmogorov Smirnov (K-S) test. All statistical analyses were conducted using SPSS 19 with an alpha criterion of $p<0.05$.

3.4 Results

3.4.1 Structural analysis

Examination of all 40 brains under light microscope confirmed the presence of bilateral nodular heterotopia in all 20 mutant subjects (10 male, 10 female), and absence of heterotopia in all 20 WT subjects (10 male, 10 female). Detailed descriptions of the malformations (i.e., heterotopia volume and estimates of neuron number) have been reported elsewhere for this strain (Rosen et al., 2013; Truong et al., 2013). Importantly, no sex differences in heterotopia volume or neuronal population were observed (Truong et al., 2013). Initial examination of stereological data was conducted for left and right structures (MGN, VCN, DCN) separately, using Hemisphere as a within-subject variable. However, no main effects of Hemisphere (or interactions with Hemisphere) were found across neuronal cell population or overall cell size for any structure, therefore subsequent analysis combined left and right optical fractionators and nucleator data for each structure into a single total measure.

Initial analysis for normality using Shapiro-Wilk test on cumulative percent distributions of cell size within the MGN, VCN, and DCN were separately conducted across both Strain and Sex. Results from this test were statistically significant ($p<0.001$

for all measures), indicating that cell size distribution data across Strain and Sex did not follow a normal distribution, and thus further statistical analysis of cumulative percent distributions were undertaken using non-parametric analyses (Kruskal-Wallis and K-S test).

3.4.2 BXD29-Tlr4^{lps-2J}/J mutant mice had smaller neurons in the MGN

A univariate ANOVA comparing total neuronal cell counts in the MGN, with between-subject parameters of Strain (2 levels: Mutant and WT) and Sex (2 levels), revealed no main effects of Strain *[F(1,36)=1.02, N.S.]*, Sex *[F(1,36)<1, N.S.]*, nor Strain x Sex interaction *[F(1,36)=2, N.S.]* (Figure 3.1.A). However, analysis of mean cell size in the MGN did reveal a main effect of Strain *[F(1,36)=15.62, p<0.001]*, but no main effect of Sex *[F(1,36)<1, N.S.]* or Strain × Sex interaction *[F(1,36)<1, N.S.]*. This main effect reflected the fact that mutant mice (regardless of sex) had a mean difference in cell size, with smaller neurons in the MGN in comparison to coisogenic WTs (Figure 3.1.B).

Omnibus nonparametric analysis of cumulative percent distribution across both Strain and Sex using the Kruskal-Wallis test further revealed significant differences in median cell size across groups (WT female Mdn = 90.53 μm^2; WT male Mdn = 91.18 μm^2; Mutant Female Mdn = 80 μm^2; Mutant Male Mdn = 77.35 μm^2; $\chi^2(3) = 904$, $p<0.001$). To determine which group(s) contributed to significance, *post hoc* analyses examined pairwise comparisons within Sex, specifically comparing mutant and WT cumulative percent distributions. Comparison within females revealed a significant difference in median cell size *[$\chi^2(1) = 273$, $p<0.001$]*, with follow-up analysis (K-S test) showing a significant difference in cell size distribution ($p<0.001$). Both results indicate a

shift in the distribution toward smaller neurons in the MGN of female mutant brains in comparison to WTs (Figure 3.1.C). Examination of male brains revealed a similar pattern of results, with *post hoc* analysis showing a significant difference in median cell size between mutant and WT males *[$\chi^2(1) = 632$, $p<0.001$]*, and follow-up analysis (K-S test) showing a significant shift in male mutant cell size distribution toward more small neurons (*$p<0.001$*; Figure 3.1.D). Taken together, overall analysis of cumulative percent distribution of cell sizes indicated that mutant subjects of both sexes have smaller neurons in the MGN in comparison to wild types.

3.4.3 BXD29-Tlr4^{lps-2J}/J mutant females had changes in cell size distribution in the VCN

Analysis of total neuron cell count in the VCN via univariate ANOVA showed no main effect of Strain, Sex, or Strain × Sex interaction *[$F(1,36)<1$, N.S., all variables]*, indicating no differences in neuron numbers across groups (Figure 3.2.A). In addition, comparison of mean cell size in the VCN across groups via a univariate ANOVA also revealed no differences in mean neuronal size (Figure 3.2.B).

Overall analysis of cumulative percent cell size distribution in the VCN across both Strain and Sex did, however, reveal a significant difference of median cell size using the Kruskal-Wallis test (WT female Mdn = 109.58 μm^2; WT male Mdn = 110 μm^2; Mutant Female Mdn = 115.75 μm^2; Mutant Male Mdn = 108.91 μm^2; *$\chi^2(3) = 58.03$; $p<0.001$*). *Post hoc* analysis within females only revealed a significant difference of median cell size between mutant and WT *[$\chi^2(1) = 42.34$, $p<0.001$]*, with comparison of cell size distribution (K-S test) also showing a significant difference in distributions

($p<0.001$) (Figure 3.2.C). Interestingly, this statistic indicated a shift to *larger* cells in the VCN of mutant females. Analysis in male brains did *not* reveal a significant difference in median cell size between mutant and WT male brains *[$\chi^2(1) = 2.42$, N.S.]* (Figure 3.2.D). Combined results show that a shift in cell size distribution specifically in female mutant brains contributed to the omnibus Kruskal-Wallis effect, with a shift toward a greater percentage of *larger* cells as compared to small cells in the VCN of this group.

3.4.4 Altered neuronal morphology in DCN of BXD29-Tlr4^{lps-2J}/J mutant females

Comparison of total cell counts in the DCN showed no differences in neuron number across Strain *[$F(1,36)<1$, N.S.]* or Sex *[$F(1,36)=3.2$, N.S.]*, nor did we see a Strain × Sex interaction *[$F(1,36)<1$, N.S.]* (Figure 3.3.A). Mean cell size analysis further showed no main effect of Strain *[$F(1,36)=2.32$, N.S.]* or Sex *[$F(1,36)=1.34$, N.S.]*, but a significant Strain × Sex interaction was observed *[$F(1,36)=5.22$, $p<0.05$]* (Figure 3.3.B). Additional *post hoc* analysis using Fisher's LSD determined that the significant interaction was pulled by a significant difference in the mean DCN cell size of mutant female brains, with larger overall cell size in comparison to WT female brains.

Subsequent nonparametric analysis of cumulative percent cell size distribution across all groups revealed a significant overall Kruskal-Wallis effect indicating differences in median cell size (WT female Mdn = 92.2 μm^2; WT male Mdn = 96.32 μm^2; mutant female Mdn = 96.52 μm^2; mutant male Mdn = 93.57 μm^2; *$\chi^2(3) = 11.1$, $p<0.01$*). Follow-up *post hoc* analysis within each Sex indicated a difference in median cell size between mutant and WT female brains *[$\chi^2(1) = 7.79$, $p<0.01$]*, as well as a significant difference in distributions *($p<0.01$)* (Figure 3.3.C). Comparison of median cell

size between male mutant and WT brains showed no differences, however *[$\chi^2(1) = 3.19$, N.S.]* (Figure 3.3.D). Based on these results, mutant female brains had a significant rightward shift toward *large* cells than small in the DCN relative to female WTs, an effect that was not observed in males.

3.5 Discussion

In the current study, analysis of mean MGN cell size revealed significantly smaller neurons in the MGN of BXD29-*Tlr4*$^{lps-2J}$/J mutant mice of both sexes. In addition, following assessment of cell size distributions in each group, we found that mutant brains also had relatively more small neurons and fewer large neurons in the MGN to BXD29/Ty coisogenic controls (WT). These findings are consistent with prior behavioral characterization of these same male and female BXD29-*Tlr4*$^{lps-2J}$/J mutant mice (from whom brain tissue was obtained), with both male and female mutants demonstrating a severe auditory processing impairment despite normal hearing ability (comparable to WTs) on a baseline auditory control task (Truong et al., 2013). Specifically, mutant subjects were unable to behaviorally discriminate breaks in continuous white noise shorter than 75 ms in duration, while WTs were capable of detecting silent gaps as short as 2 ms in duration. These behavioral results showed that mutant mice could not perform the silent gap detection task as proficiently as WT (Truong et al., 2013). In addition to these anatomic MGN findings, we observed sex differences in cellular changes within the VCN and DCN of mutant mice. Collective results are discussed further below.

3.5.1 MCD and "magnocellular" anomalies

As previously described, MCD have been associated with language and reading disorders in both *post mortem* and *in vivo* assessments of affected individuals. Sensory processing impairments in visual and auditory modalities have also been reported in reading and language disabled populations, but impairments observed were not generalized. In the visual domain, deficits found in dyslexic individuals were specific to rapidly presented/low contrast stimuli (Livingstone et al., 1991). These patterns of deficits were consistent with processing related to the magnocellular system—a subdivision of the lateral geniculate (visual) thalamic nucleus (LGN) consisting of large neurons, and functionally involved in the processing of rapidly changing/low contrast stimuli (Livingstone & Hubel, 1988). This system complements the parvocellular system of the LGN, which is characterized by smaller neurons, and functionally is involved in the processing of slow, high contrast information (Livingstone & Hubel, 1988). Within the auditory domain, processing deficits seen in language disabled populations are also specific to rapidly presented stimuli (Benasich & Tallal, 2002; Boscariol et al., 2010b; Cohen-Mimran & Sapir, 2007; Hari & Renvall, 2001; Raschle et al., 2013; Tallal & Piercy, 1973; Tallal, 1980; Tallal & Newcombe, 1978). However, it must be noted that there is no clear magnocellular subdivision within the auditory thalamic nucleus, as seen in the visual system. Interestingly, neuromorphometric analysis of *post mortem* brain tissue of dyslexic individuals revealed smaller cell size in the magnocellular division of the LGN (Livingstone et al., 1991), and subsequent examination of the MGN also revealed more small and fewer large cells in dyslexic individuals as compared to non-reading impaired controls (Galaburda, Menard & Rosen, 1994). These findings are

consistent with rodent models of injury-induced and genetic models of MCD, where impairments in RAP—coupled with a shift in cell size distribution towards more small cells in the MGN—have both been reported relative to controls (Herman et al., 1997; Rosen et al., 2006; Rosen, Herman & Galaburda, 1999; Szalkowski et al., 2013). In the context of the current study and the mouse model of MCD discussed here, our stereological results demonstrating a shift in cell size toward smaller neurons in the MGN, coupled with impaired auditory processing in mutant mice, correspond nicely to the collective findings described above.

Although the present results further support a link between MCD, impaired auditory processing, and alterations in MGN morphology, the precise neural underpinnings of the deficient auditory phenotype as well as the etiology of the thalamic alterations remain unclear. Atypical neuronal connectively has widely been related to MCD, with evidence from rodent models identifying abnormal cortico-thalamic/thalamo-cortical connectivity in cortices where cortical malformations were located (Goldman & Galkin, 1978; Jenner, Galaburda & Sherman, 2000; Rosen, Burstein & Galaburda, 2000). However, the location of heterotopia in BXD29-*Tlr4*$^{lps-2J}$/J mutant mice are subcortical and bilateral, and located between the retrosplenial cortex and the somatosensory and visual cortices (Rosen et al., 2013). Physically, these malformations are generally not in close proximity to the auditory cortex (where the MGN has direct projections), but this does not necessarily imply that malformations do not disrupt afferent and/or efferent projections associated with auditory processing. Indeed, early neural disruptions in brain development (such as heterotopia formation) are likely to have far-reaching effects on distal brain networks. Nonetheless, how the heterotopia precisely alters neuronal

connectivity, if at all, remains unclear. In addition, mutant mice also present with partial callosal agenesis in the caudal regions of the corpus callosum, further supporting the potential for altered cortical connectivity within the brain (Rosen et al., 2013). Given behavioral and anatomical evidence in BXD29-*Tlr4*$^{lps-2J}$/J mutant mice suggesting atypical neural networks, future tracing studies will be needed to determine whether the cells within the heterotopia participate in aberrant interactions, and also how connectivity may be altered between auditory cortex and MGN (among other networks).

3.5.2 Sex differences in anatomic anomalies

A particular interesting finding within the current set of stereological data was the observation of sex differences in anomalies of the VCN and DCN, but not the MGN, of BXD29-Tlr4^{lps-2J}/J mutant mice. The lack of sex differences in MGN cell size and distribution were not surprising given that both male and female BXD29-*Tlr4*$^{lps-2J}$/J mice showed comparable severe impairments in auditory processing (Truong et al., 2013). However, sex differences in both VCN and DCN cell sizes specific to mutant brains were unexpected, given the uniform deficit in auditory processing observed for mutant mice of both sexes. Further *post hoc* analysis suggested that sex differences in mutant subjects were driven by more *large* cells in the VCN and DCN of mutant females, while mutant male brains showed VCN and DCN cellular morphology comparable to matched WTs. These findings raise an interesting, yet puzzling question: why do anatomical changes in the VCN and DCN only occur in female but not male mutant brains, while both sexes show a similar distribution shift to small cells in the MGN? Also, how do these findings relate to evidence of a similar behavioral deficit profile in mutant males and females?

Some prior research has addressed abnormal cellular morphology in the cochlear nucleus. For example, extensive work on the CN in gerbils explored the highly plastic nature of neurons within this region based on afferent connections between the cochlea and VCN via the auditory nerve (Pasic & Rubel, 1989; Pasic & Rubel, 1991; Pasic, Moore & Rubel, 1994; Sie & Rubel, 1992). Investigators found that when the auditory nerve was transiently inactivated for 24–48 hours using Na+ channel blocker, tetrodotoxin (TTX), VCN cell size was reduced as much as 25% in as little as 24 hours following auditory nerve inactivation (Pasic, Moore & Rubel, 1994; Sie & Rubel, 1992). A week of typical auditory nerve innervation of the VCN following the 48 hr blockade revealed full recovery of normal neuronal cell size in the VCN. This suggests that morphological changes in the CN may be regulated by activity-dependent processes.

In the case of BXD29-*Tlr4*$^{lps-2J}$/J mutant females, one potential explanation for the increase in cell size in both the VCN and DCN could be sex-dependent alterations in afferent input to the CN. Interestingly, both the VCN and DCN subdivisions of the CN receive direct innervation from primary auditory cortex (A1) via corticofugal projections from layer V of the cortex—a pathway that appears ethologically consistent among several species including guinea pigs (Jacomme et al., 2003; Schofield, Coomes & Schofield, 2006), rats, and mice (Meltzer & Ryugo, 2006). This direct projection from A1 to CN implies a modulation of CN activity via higher-order structures. Thus based on the known activity dependent changes in CN cell morphology, it may be that changes in neuronal connectivity between A1 and CN (as well as other afferent inputs to the CN offering sensory feedback) could in turn mediate changes in CN cell size. However, this

suggestion of A1 modulation of CN neuronal plasticity is speculative, and no work has been conducted to test this conjecture.

Although not directly related to A1-CN connectivity and plasticity, a finding of larger cells in the MGN, coupled with RAP impairments, have been reported previously. Specifically, in studies of a rodent model of hypoxia ischemia (HI; where disruption of cortical development occurs as a result of early postnatal HI injury to the brain), investigators found that rats with a postnatal day 7 (P7) HI injury showed a shift toward more large and fewer small cells in the MGN contralateral to the side of injury (whereas a shift towards smaller cell size was seen in the MGN on the side of injury; Alexander et al., 2014). In addition, P7 HI injured male rats also showed impairments in RAP (note, females subjects were not assessed in these studies; Alexander et al., 2014). The presence of more *large* cells in the P7 HI model suggests that developmental compensation may have occurred as a result of HI injury (Johnston, 2009), although apparently insufficient to ameliorate the sensory processing deficit observed. Additional studies must be conducted to further explore the effects of early HI injury on subcortical developmental and compensation/re-organization, using this or related rodent models of developmental neural disruption.

Alternatively, sex differences in the development of CN morphology of BXD29-*Tlr4*$^{lps-2J}$/J mutant brains could also be explained by potential sex-specific genetic and/or hormonal mechanisms. Specifically, injury-induced models of altered cortical development have revealed sex specific differences in both behavioral and neuroanatomical outcome, and these effects have been associated with both genetic and hormonal manipulations (see Hill & Fitch, 2012, for review). In BXD29-*Tlr4*$^{lps-2J}$/J

mutant mice, the etiology of the severe neuroanatomical phenotype is still under investigation, however, the 100% penetrant nature of the malformations point to a genetic cause (Rosen et al., 2013). It may be that the genetic mechanism(s) related to the disruption of cortical development and leading to the cortical malformations may also play a role in regulating cell morphology in other structures (e.g., the MGN and CN). Furthermore, these genetic mechanisms may also participate in sexually dimorphic pathways that could ultimately lead to differing development of these structures—a conjecture that is supported by literature showing sex specific differences in brain morphology within some clinical populations (Evans et al., 2014; Galaburda et al., 1985; Goldstein et al., 2002; Humphreys, Kaufmann & Galaburda, 1990; Lai et al., 2013).

Although the results surrounding the sexually dimorphic changes in cell size within the CN of mutant BXD29-*Tlr4*$^{lps-2J}$/J brains are puzzling, these findings are not the first to describe sex differences in neuroanatomical anomalies following disruption of cortical development. In actuality, numerous studies have reported structural differences in the brain in relation to sex within clinical and rodent model literature (Evans et al., 2014; Galaburda et al., 1985; Goldstein et al., 2002; Herman et al., 1997; Hill, Threlkeld & Fitch, 2011; Hill et al., 2011; Humphreys, Kaufmann & Galaburda, 1990; Lai et al., 2013; Lan et al., 2011; Rosen et al., 2006; Rosen, Herman & Galaburda, 1999). In an HI injury model, it was found that male HI injured rats showed impairments on a RAP task, while comparably injured females showed similar RAP performance to controls (Hill et al., 2011; Hill, Threlkeld & Fitch, 2011). Behavioral and anatomical characterization of rats with injury induced cerebrcortical microgyria also found RAP impairments in male microgyric rats, as well as smaller cells in the MGN (Fitch et al., 1997; Fitch et al., 1994;

Herman et al., 1997; Rosen et al., 2006), but no evidence of deficits in RAP or cell size changes in the MGN were seen in comparably injured female microgyric rats (Fitch et al., 1997; Peiffer, Rosen & Fitch, 2004). Given that sex differences in structural changes to the brain associated with disruption of cortical development have been widely reported, it remains unclear how these differences manifest in a behavioral phenotype, especially in the present study. Although BXD29-*Tlr4*$^{lps-2J}$/J mutant females show larger cells in the CN, no sex differences in performance on the specific auditory processing tasks used were observed. However, that does not necessarily suggest that a sex difference in the auditory domain is absent, and it may be that assessment on a different type of auditory processing task (i.e. sound localization in contrast to RAP) may elicit an associated sex difference in behavior.

3.6 Conclusion

Histological examination of the MGN, VCN, and DCN in a BXD29-*Tlr4*$^{lps-2J}$/J mouse mutant with previously characterized MCD and a severe RAP deficit phenotype did not reveal changes in neuron numbers across strain or sex. However, further stereological assessment of these structures *did* find mutant-specific changes in the MGN revealing not only overall smaller cells, but also more small and fewer large cells in the MGN, regardless of sex. Interestingly, a sexually dimorphic shift to larger cells in both the VCN and DCN were observed in mutant females only. These findings support a link between MCD, impaired auditory processing behaviors, and alterations in MGN morphology, with the current series of assessments extending these findings to cellular changes in the CN. Given the current data-set, although the behavioral etiology of the RAP impairment in BXD29-*Tlr4*$^{lps-2J}$/J mutant mice remain unclear, this new evidence can provide a

platform for further experimentation to investigate how neuronal connectivity may be altered as a result of MCD, and whether physiologic anomalies may manifest to explain deficiencies in auditory processing behavior. This series of stereological results also highlights sex differences in subcortical anatomy related to MCD. Again, further experimentation must be conducted to determine how structurally deviant development occurs, and whether unique behavioral correlates of these cellular changes in male and female mutant mice can be identified.

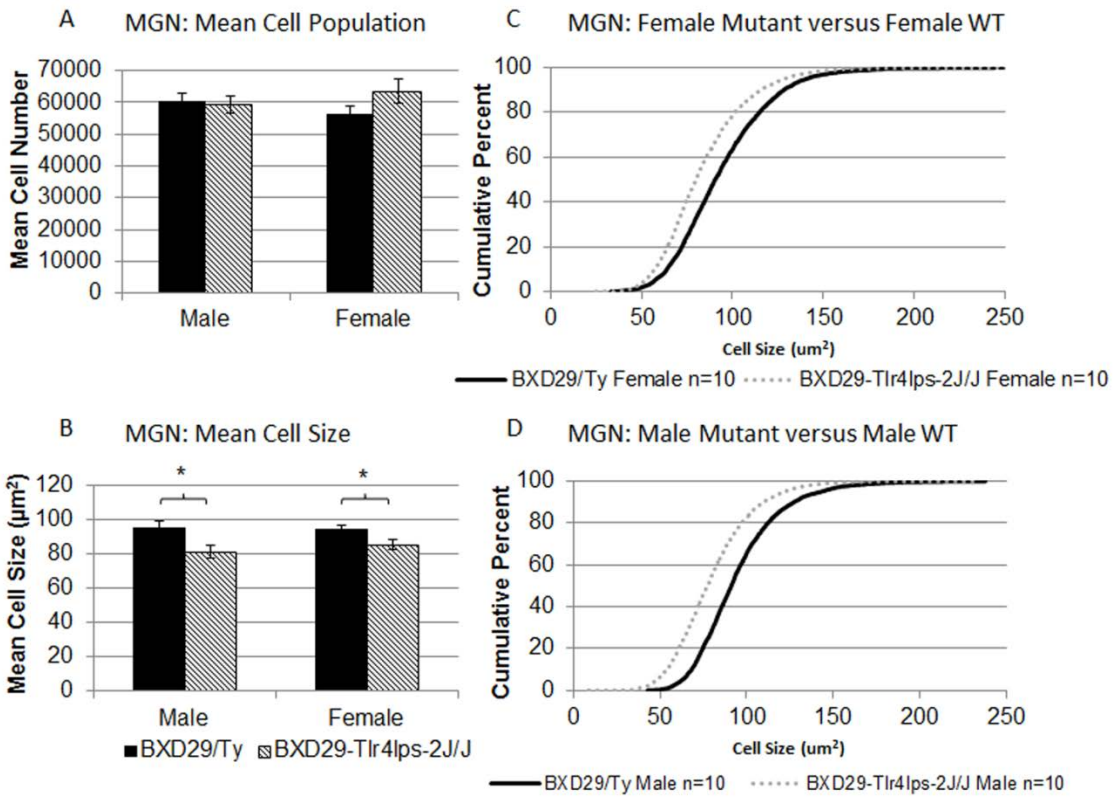

Figure 3.1 Stereological assessment of the medial geniculate nucleus (MGN) in BXD29-*Tlr4*$^{lps-2J}$/J mutant and BXD29/Ty strains of mice. (A) Estimates of total cell counts in the MGN show no difference in neuronal population across all groups. (B) Comparison of mean cell size in the MGN revealed a significant Strain effect, indicating that mutant mice had overall smaller neurons in the MGN in comparison to WT controls. This effect was observed regardless of sex. Analysis of cumulative percent cell size distribution showed a significant shift in cell size toward more small cells in the MGN in both female (C) and male (D) BXD29-*Tlr4*$^{lps-2J}$/J mutant brains. *$p<0.05$.

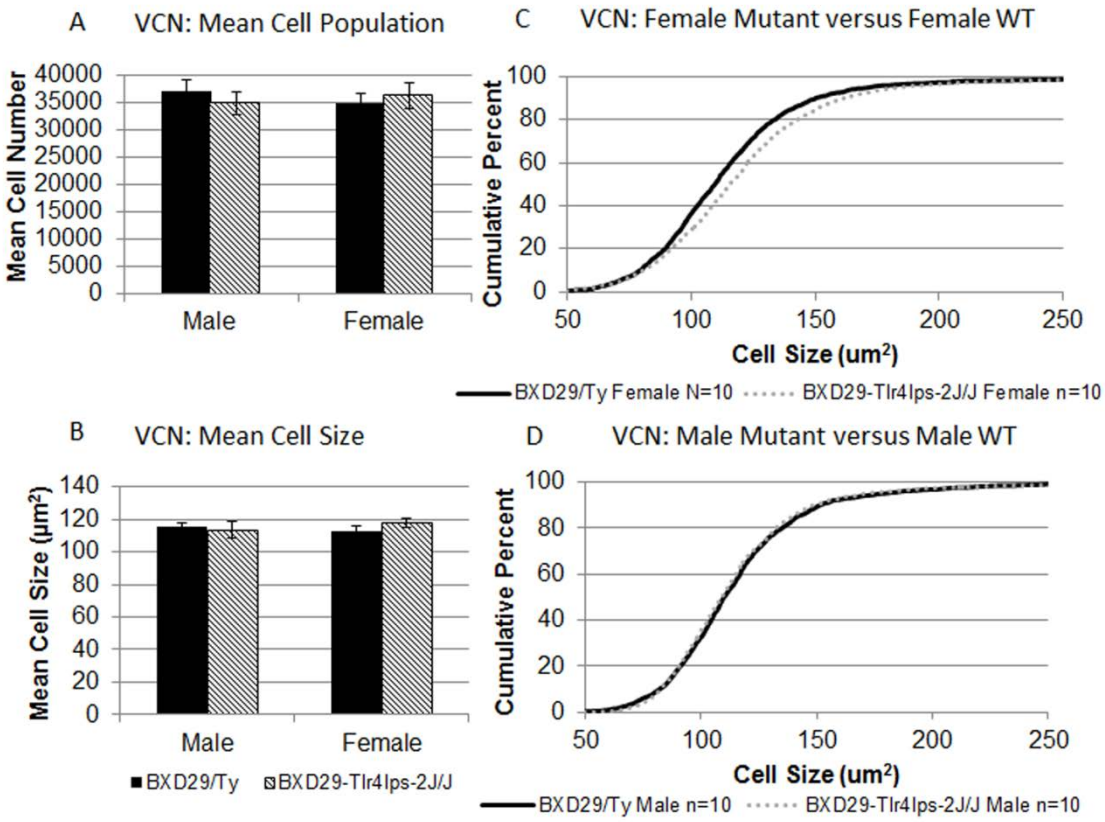

Figure 3.2 Stereological examination of the ventral cochlear nucleus (VCN) in BXD29-*Tlr4*$^{lps-2J}$/J mutant and BXD29/Ty WT strains of mice. (A) Analysis of total neuronal cell count estimates and (B) mean cell size revealed no significant differences across Strain or Sex. (C) Examination of the cumulative percent distributions of neuronal cell size in the VCN found a significant difference in the distributions between female mutant and WT brains. Specifically, mutant female brains showed a shift toward more large neurons in the VCN as compared to small neurons. (D) The same analysis conducted in males yielded no differences in cumulative percent cell size distribution between mutant and WT strains. Cell distribution analysis revealed sexually dependent changes in the VCN of BXD29-*Tlr4*$^{lps-2J}$/J mutant brains.

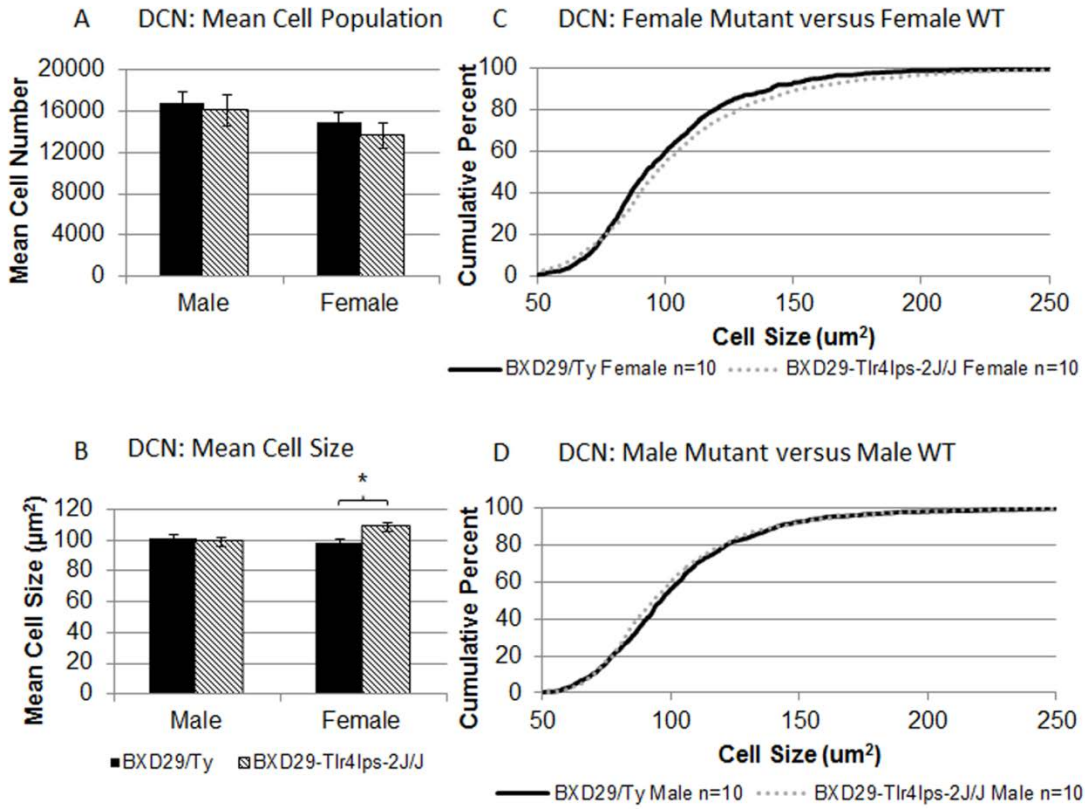

Figure 3.3 Stereological assessment of the dorsal cochlear nucleus (DCN) in BXD29-*Tlr4*$^{lps-2J}$/J mutant and BXD29/Ty strains of mice. (A) Analysis of total estimated cell count in the DCN found no differences in neuronal population between mutant and WT brains. (B) Assessment of mean cell size did find a sex difference between male and female mutant mice, indicating significantly larger overall cell size in the DCN of female mutant brains in comparison to WT. No differences in mean cell size were observed between male mutant and WT brains. (C) Examination of cumulative percent distribution of cell size found a similar sexually dimorphic pattern, with mutant females showing a significant shift toward more large neurons in the DCN relative to female WT. (D) Both male mutant and WT brains showed no differences in cell size distribution, thus indicating sex differences in DCN cellular morphology of BXD29-*Tlr4*$^{lps-2J}$/J mutant brains. *$p<0.05$

CHAPTER 4

Mutation of *Dcdc2* in mice leads to impairments in auditory, somatosensory, and memory processing

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4.1 Abstract

Dyslexia is a complex neurodevelopmental disorder characterized by impaired reading ability despite normal intellect, and is associated with specific difficulties in phonological and rapid auditory processing (RAP), high-frequency tactile discrimination, visual attention, and working memory. Genetic variants in *DCDC2* have been associated with dyslexia, impairments in phonological processing, and in short term/working memory. The purpose of this study was to determine whether sensory and behavioral impairments can result directly from mutation of the *Dcdc2* gene in mice. Several behavioral tasks, including a modified pre-pulse inhibition paradigm (to examine auditory processing), a 4/8 radial arm maze (to assess/dissociate working versus reference memory), rotarod (to examine sensorimotor ability and motor learning), and a modified novel object recognition task (to examine tactile discrimination) were used to assess the effects of *Dcdc2* mutation. Behavioral results revealed deficits in RAP, working memory and reference memory, and tactile discrimination in $Dcdc2^{del2/del2}$ mice as compared to matched wild types. Current findings parallel clinical research linking genetic variants of *DCDC2* with specific impairments of phonological processing and memory ability.

4.2 Introduction

DCDC2 (Doublecortin domain containing protein 2; chromosome 6p22) was identified as a candidate gene for dyslexia, a multifactorial neurobehavioral disorder associated with multiple gene and environmental risk factors (Meng et al., 2005). Expressed in neural areas critical to reading (e.g., inferior and medial temporal cortex; Cope et al., 2012; Meng et al., 2011), *DCDC2* variants have also specifically been linked to behavioral deficits related to reading disability, including phonological processes, as well as single letter forward and backward span tasks (short term and working memory, respectively; Berninger et al., 2008; Marino et al., 2011b). Overall, several independent research groups have associated *DCDC2* with impaired reading related measures in divergent populations (Lind et al., 2010; Marino et al., 2011b; Meng et al., 2005; Powers et al., 2013; Scerri et al., 2011; Schumacher et al., 2006).

Although the molecular function of *DCDC2* remains unclear, clues to its neurobiological function have been inferred through its relationship to the *DCX* superfamily of genes that contain doublecortin peptide domains, and are related to microtubule organization and assembly (Coquelle et al., 2006). In addition, *DCX* is a widely studied gene known for its function in neuronal migration and cortical development (Gleeson et al., 1999). To further examine the function of *DCDC2* and its potential role in neuronal migration, studies using *in utero* electroporation of RNAi against *Dcdc2* in rats revealed that neurons transfected with *Dcdc2* RNAi migrated a shorter distance from the ventricular zone compared to neurons transfected with control plasmid (Burbridge et al., 2008; Meng et al., 2005). In addition, *DCDC2* protein is localized in primary cilia of rat primary hippocampal neurons with alterations in

expression leading to changes in primary cilia length and cell signaling—processes critical to appropriate neuronal migration and cortical development (Lee & Gleeson, 2010; Massinen et al., 2011). These findings suggest that *Dcdc2* plays a key role in early brain development. Interestingly, studies of *Dcdc2* knockout mice (*Dcdc2*$^{del2/del2}$) reveal no gross neuromorphological anomalies or evidence of migrational disruption (Wang et al., 2011). Nonetheless, neurophysiological recordings of layer 2/3 and layer 4 pyramidal neurons in somatosensory cortex of mutant mice revealed that *Dcdc2* is required to maintain normal neuronal excitability and temporally precise patterns of action potential firing rates (Che, Girgenti & LoTurco, 2013).

With regard to specific behavioral assessments of *Dcdc2*$^{del2/del2}$ mice, one report using a modified Hebb-Williams maze and visual discrimination task reported that subjects were unimpaired when tested on "easier" cognitive tasks. However, a visuo-spatial learning/memory impairment emerged when *Dcdc2*$^{del2/del2}$ mice were required to perform more cognitively demanding tasks (i.e., more complex Hebb Williams maze configurations and longer inter-trial intervals; Gabel et al., 2011).

The current series of experiments were designed to examine the role of *Dcdc2* function using behavioral paradigms that have been adapted to model basic nonverbal behaviors that may be dysfunctional in language and reading impaired populations—specifically auditory processing, working and reference memory ability, and motor learning. Given recent evidence that *Dcdc2*$^{del2/del2}$ in mice exhibit atypical neuronal spike timing (indicating imprecise temporal encoding of input), along with human findings implicating *DCDC2* in memory ability and phonological processing, we predicted that

behavioral assessment of *Dcdc2*$^{del2/del2}$ mice would yield evidence of impairment on some/all tasks.

4.3 Materials and Methods

4.3.1 Subjects

Dcdc2 knockout mice (*Dcdc2*$^{del2/del2}$) carried a constitutive homozygous deletion of exon 2 (del2) within the *Dcdc2* gene region of a 129SJ x C57BL/6J hybrid background backcrossed to C57BL/6J for 10 generations (see Wang et al., 2011, for detailed *Dcdc2* gene targeting and RT-PCR analysis). All subjects were generated from the *Dcdc2* colony maintained by AC/JJL at the University of Connecticut using a heterozygous-heterozygous (*Dcdc2*$^{wt/del2}$ × *Dcdc2*$^{wt/del2}$) mating scheme with resultant genotypes recovered in the expected mendelian ratios (1:2:1). Two separate cohorts of *Dcdc2*$^{del2/del2}$ and *Dcdc2* wild type (WT) were generated from these breedings. Cohort 1 included 6 WT and 13 *Dcdc2*$^{del2/del2}$ mice, with behavioral assessment starting at 20 weeks. Cohort 2 included 11 WT and 9 *Dcdc2*$^{del2/del2}$ mice, with behavioral assessment starting at 11 weeks. Only male subjects were assessed across behavioral measures. All subjects were single-housed in standard mouse tubs (12 h/12 h light/dark cycle), with food and water *ad lib.* In addition, all behavioral testing occurred during the light cycle. Procedures were performed blind to genotype and in compliance with the National Institutes of Health and University of Connecticut's Institutional Animal Care and Use Committee (IACUC).

4.3.2 Auditory Processing—Silent Gap and Embedded Tone

Subjects in cohorts 1 and 2 (17 WT and 22 *Dcdc2*$^{del2/del2}$ mice) were examined for auditory processing ability using a modified pre-pulse inhibition paradigm (see Fitch et

al., 2008b, for review; Figure 4.1.A). Subjects were placed on individual load-cell platforms (MED Associates, St. Albans, VT) and presented with auditory stimuli generated using RPvdsEx on a Dell Pentium D PC and RX6 multifunction processor (Tucker Davis Technologies, Alachua, FL). Sounds were amplified using a Niles SI-1260 Integration Amplifier (Niles Audio Corp., Carlsbad, CA) and delivered through powered Yamaha YHT-M100 speakers (Buena Park, CA). The acoustic startle reflex (ASR; a reflexive response elicited by an unexpected, intense stimulus) was recorded on an iMac 7.1 running Acknowledge 4.1 and obtained via the voltage output from each load cell platform through a linear amplifier (PHM-250U; Med Associates, St. Albans, VT) connected to a Biopac MP150 acquisition system (Biopac Systems, Goleta, CA). The modified pre-pulse inhibition paradigm measured differences in ASR to a loud startle eliciting stimulus (SES) when presented with/without a preceding acoustic cue. The ASR difference on cued versus uncued trials provided a measure of cue detection and/or discrimination. If the auditory cue was detected, a reduction (attenuation) in the ASR was expected relative to the ASR elicited when the auditory cue was not present (or not detected). This phenomenon was quantified using an “attenuation score” (ATT) that compared the average amplitude of the ASR from the cued trial to the average ASR of the uncued trial ([average cued ASR/average uncued ASR]*100). First, a Silent Gap (SG) task was used to assess the ability to detect breaks in continuous white noise (Figure 4.1.B). A session included 300 trials with a continuous 75 dB broadband white noise background. Cued and uncued trials occurred pseudorandomly. On cued trials, a silent gap of variable duration (2–100 ms) was presented 100 ms before the SES (50 ms, 105 dB, white noise burst). Uncued trials lacked a silent gap cue (0 ms). Next, the variable

duration Embedded Tone (EBT) task was administered (with 300 sequential pseudorandom trials), but this task assessed ability to detect a change in tone frequency relative to a standard background tone (Figure 4.1.C; cue was a variable duration 5.6 kHz pure tone embedded in a 10.5 kHz background pure tone). On cued trials, the cue was presented 100 ms before the SES (50 ms, 105 dB), while uncued trials used a tone "cue" of 0 ms. Two EBT tasks were used in this study—a long-duration EBT (0 ms to 100 ms), and a short-duration EBT (0 ms to 10 ms).

4.3.3 Water maze assessment—Visible platform and 4/8 radial water maze

Subjects in Cohort 2[1] (11 WT and 9 *Dcdc2*$^{del2/del2}$ mice) were tested on a visible platform control task (also called "water escape") prior to radial water maze assessment, to ascertain any underlying impairments that might confound further maze testing (i.e., deficits in motivation, swimming, or visual acuity). Subjects were placed in the far end of an oval tub (103 cm × 55.5 cm) filled with room temperature water, and were given 45 seconds to swim to a visible escape platform (8.5 cm in diameter; 1 cm above water surface) located at the opposite end of the tub. Swim latencies were recorded for assessment. One *Dcdc2*$^{del2/del2}$ mouse was dropped due to impaired swimming ability. Remaining subjects (11 WT and 8 *Dcdc2*$^{del2/del2}$ mice) were then tested on a water version of the 4/8 radial arm maze (adapted from Hyde, Hoplight & Denenberg, 1998). This task measured spatial reference and working memory ability simultaneously, using a standard 8 arm radial maze with 4 arms baited (i.e., containing submerged goal platform), and 4 arms open but never baited (Figure 4.2). Configurations of goal and start arm locations

[1] Cohort 1 subjects were tested for 12 weeks on a delayed match to sample radial arm maze that proved too difficult and no subjects evidenced learning, so the data was not reported.

remained fixed across all test sessions. In addition, high contrast extra maze cues were present around the maze. Locations for these cues remained static for the entire experiment.

The day prior to testing (Day 1), subjects were given a training session where they were released from the start arm and allowed 120 seconds or 10 incorrect arm entries (errors) to locate one of four hidden goal platforms. If the subject failed to find a platform in this window, they were guided to the nearest available goal. Once on the platform, subjects remained on the platform for 20 seconds and then removed from the maze to a heated home cage (30 second inter-trial interval; ITI). During the ITI, the recently located platform was removed and the entrance to that arm was blocked so that the subject could no longer enter for the remainder of the training session. On trial 2, the subject was then placed back in the start arm, and this procedure was repeated until all four platforms were located (four trials total).

Testing began on Day 2, and continued for an additional 14 consecutive days (15 total days including training). The test session followed training procedures, but instead of blocking the goal arm of the most recently located platform, the goal platform was removed during the 30 second ITI, with the arm remaining open and unbaited for the remainder of the test session. Test sessions were recorded using a Sony camera integrated with the SMART video tracking program (Panlab, Barcelona, Spain). An arm entry was counted for a subject when all four paws entered an arm. Three types of errors were quantified for analysis. 1) Working memory errors consisted of the number of initial and repeat entries into arms from which a platform had been removed during that test session. 2) Initial reference memory errors consisted of the total number of first entries into arms

that never contained a goal platform. 3) Working memory incorrect errors were the total number of repeat entries (following the initial entry) into arms that never contained escape platforms. Total errors per test session in each category were tabulated, averaged within genotype, and used for analysis across days of testing.

Finally, in order to determine whether subjects utilized a spatial or chaining (swimming to successive adjacent arms) strategy to solve the water maze, angles of arm choices were analyzed. Video tracking data obtained from the SMART system was reviewed and turn angle entry was tabulated to determine 1) the percentage of each angle type per session and 2) the average turn angle utilized across sessions. A higher percentage of 45° turns suggest that subjects preferred adjacent arm choices to solve the maze. Alternatively, higher percentage of turns 90° and greater and/or an average turn angle around 90° would suggest a preference for more spatial strategies to solve the maze.

4.3.4 Sensorimotor/motor learning

Subjects from Cohort 2 (11 WT and 9 *Dcdc2*$^{del2/del2}$ mice) were assessed for sensorimotor ability and motor learning using the rotarod task. Subjects were placed on a rotating cylindrical drum that gradually accelerated from 4 to 40 rotations per minute across a span of 2 minutes. Four trials were administered per test day across five consecutive days. For analysis, latency to fall from the rotating drum was measured and averaged across the four trials for each day.

4.3.5 Tactile Discrimination

Subjects from Cohort 2 (11 WT and 9 *Dcdc2*$^{del2/del2}$ mice) were tested on a tactile discrimination task adapted from Wu et al., (2013), based on general principles of the novel object recognition task (using vibrissal rather than visual input). This task assessed whether subjects could detect differences in tactile frequency gradients of sandpaper. The test apparatus was a (40 cm × 24 cm × 20 cm) plexiglas tub with opaque walls. The target "objects" were 5 cm × 7.5 cm sheets of aluminum oxide sandpaper (3M, St. Paul, MN) affixed to the lateral walls of the test chamber (right and left sides), approximately 3 cm from back corner and 2 cm above the floor of the chamber. Two different grades of sandpaper were used for the texture discrimination task; 80 and 180 grade (average particle width of 190 μm and 82 μm, respectively).

For the tactile discrimination task, the following experimental protocol was used (Figure 4.3). One day prior to testing, subjects were habituated to the testing chamber for 10 minutes. On the day of testing, subjects were given one additional habituation period for 5 minutes. Immediately following, the subjects were given a 5 minute "familiarization" period were they were exposed to two identical sheets of 80 grit sandpaper, each located opposite from one another on the lateral walls. During the familiarization phase, subjects were placed in the center of the testing chamber, equidistant and facing away from the identical textured sandpaper. Following the familiarization phase, subjects were returned to their home cage for the 5 minute "resting" period. During the resting period, the textured sheets used during the familiarization phase was removed and replaced with one identical 80 grit sheet and one novel 180 grit sheet. The side with the novel and familiar object was counterbalanced

throughout testing. Following the resting period, subjects were placed back into the testing chamber for the final 3 minute “testing” period. A Sony video camera was used to record the testing period and the amount of time each subject actively interacted with each of the textured sheets was used for analysis.

Four weeks following the texture discrimination task, a visual control task was employed to insure that any differences in texture discrimination behavior could be attributed to differences in tactile processing and not visual information. Clear transparent film (Computer Grafix, Maple Heights, OH) was used to cover the textured faces of the sandpaper (making the objects texture-less), and the experimental protocol was repeated (Figure 4.3). This allowed for evaluation of the possible use of visual information by the subjects in order to discriminate between the two different grades of sandpaper.

4.3.6 Statistical Analysis

All behavioral data was analyzed using a mixed factorial design. A 2 x 9 repeated measures analysis of variance (ANOVA) with Genotype (2 levels: WT and *Dcdc2*$^{del2/del2}$) as the between measure and Gap (9 levels) as the within measure was conducted to analyze SG auditory processing performance. A 2 × 9 × 2 and 2 × 9 × 4 repeated measures ANOVA was performed on EBT 0–100 ms and EBT 0–10 ms, respectively, with Genotype (2 levels: WT and *Dcdc2*$^{del2/del2}$) as the between measure and Duration (9 levels) and Day (2 levels for EBT 0–100 ms; 4 levels for EBT 0–10 ms) as within measures. In addition, subjects were assessed for auditory cue discrimination using a paired samples t-test comparing mean cued and uncued ASR. Average total, working memory correct, reference memory, and working memory incorrect errors on the 4/8

radial arm maze were independently examined using a 2 × 14 repeated measures ANOVA, with Genotype (2 levels: WT and *Dcdc2*$^{del2/del2}$) as the between measure and Test Session (14 levels) as the within measure. Within session performance across trials was conducted using a 2 × 4 × 14 repeated measures ANOVA with Genotype (2 levels: WT and *Dcdc2*$^{del2/del2}$) as the between measure and Trial (4 levels) and Session (14 levels) as within measures. In addition, a univariate ANOVA and a paired samples t-test was used to assess between- and within-group differences, respectively, in turn angle preference and average turn angle on the 4/8 radial arm water maze. Group differences in rotarod performance were analyzed using a repeated measures ANOVA with Genotype (2 levels: WT and *Dcdc2*$^{del2/del2}$) as the between measure and Day (5 levels) as the within measure. Finally, differences in percent interaction between the novel and familiar tactile stimuli were conducted using a paired samples t-test within *Dcdc2*$^{del2/del2}$ and WT groups. All statistical analyses were conducted using SPSS 19 with an alpha criterion of 0.05.

4.4 Results

4.4.1 Dcdc2 deletion does not affect silent gap (SG) detection, but impairs tone detection

WT performance across Cohorts 1 and 2 were compared to ensure comparable performance before pooling data. Within genotype analysis across cohorts demonstrated that WT controls performed comparably on the SG 0–100 ms *[F(1,15)<1, N.S]*, EBT 0–100 ms *[F(1,15)<1, N.S.]*, and EBT 0–10 ms *[F(1,15)=3.56, N.S.]* paradigms. Thus data from cohorts 1 and 2 were pooled, for a total n of WT n=17; *Dcdc2*$^{del2/del2}$ n=22.

Subjects were initially administered a gap detection task using silent gaps between 0 and 100 ms. Performance on this task was poor (mean values for each group shown in Figure 4.4.A), and there was no overall Genotype effect *[F(1,37)=3.14, N.S]*, nor Genotype × Duration interaction *[F(8,296<1, N.S.]*. Next, we administered an embedded tone task, also at 0–100 ms durations. Overall performance on this task was better than SG (mean ATT 75 *Dcdc2*$^{del2/del2}$, 69 WT; Figure 4.4.B), and a Genotype × Duration interaction was seen *[F(8,296)=3.45, P<0.001]* with significant differences at 20 ms *(P<0.05)* and marginally significant differences at 75 and 100 ms (*Dcdc2*$^{del2/del2}$ worse than WT; *P<0.1*). However, no main effect of Genotype was observed on the EBT 0–100 ms task *[F(1,37)=2.39, N.S.]*. Finally, we performed an embedded tone task using 0–10 ms stimuli. Although this task was more difficult than 0–100 (as supported by a large psychophysical literature on gap detection in humans and rodents; see Fitch et al., 2008 for review), subjects overall performed roughly comparable to the 0–100 ms task, reflecting a counteracting contribution of ongoing experience (with experience enhancing gap acuity on this task; Fitch et al., 2008). Interestingly, on the 0–10 ms EBT task, a significant overall effect of Genotype was found, with *Dcdc2*$^{del2/del2}$ performing worse overall as compared to WT *[F(1,37)=5.86, P<0.05]* (Figure 4.4.C).

4.4.2 Dcdc2 mutant mice show impaired working and reference memory

Prior to spatial water maze testing, a visible platform control task was conducted to assess for underlying impairments that could confound water maze performance (e.g. swim, see, motivation). A univariate ANOVA on latencies found no main effect of Genotype *[F(1,17)<1, N.S.]* indicating that *Dcdc2*$^{del2/del2}$ subjects showed no impairments on underlying aspects of the task (Figure 4.5). One *Dcdc2*$^{del2/del2}$ mouse was dropped due

to impaired swimming ability, thus 11 WT and 8 *Dcdc2*$^{del2/del2}$ mice were studied for analysis.

The 4/8 radial arm water maze was used to simultaneously measure spatial working and reference memory ability. Analysis of the average number of total errors (working memory correct, initial reference, and working memory incorrect) revealed a significant difference between WT and *Dcdc2*$^{del2/del2}$ groups *[F(1,17)=8.86, P<0.01]* via repeated measures ANOVA, with *Dcdc2*$^{del2/del2}$ making significantly more errors that WTs (Figure 4.6.A). A main effect of Session *[F(12,221)=3.54, P<0.001]* was also observed, confirming that both groups reduced errors across sessions (i.e., showed learning). Within test session analysis of total errors across trials revealed a main effect of Genotype *[F(1,17)=7.42, P<0.05]* and a Trial × Genotype interaction *[F(3,51)=3.62, P<0.05]* with *Dcdc2*$^{del2/del2}$ subjects making significantly more errors on trials 2 and 4 *(P<0.05)* as more platforms were removed from the maze (Figure 4.6.B). Individual analysis of group differences for different error types was also performed, to determine whether impairments in specific memory domains (i.e., working memory correct, reference memory, and working memory incorrect) could be observed. A repeated measure ANOVA on total *working memory correct* errors (across Sessions) revealed that *Dcdc2*$^{del2/del2}$ subjects made significantly more errors than WT subjects *[F(1,17)=6.02, P<0.05]*. No main effect of Session *[F(13,221)=1.41, N.S.]* nor Session × Genotype interaction *[F(13,221)<1, N.S.]* (Figure 4.6.C) were observed, indicating that working memory errors for both groups did not significantly change over days of testing. Analysis of *reference memory* errors (repeated measures ANOVA across Sessions) found that *Dcdc2*$^{del2/del2}$ subjects made significantly more reference memory errors than WT controls

[F(1,17)=4.76, P<0.05]. Within subject analysis of reference memory errors did show a significant main effect of Session *[F(13,221)=3.54, P<0.001]* (indicating learning), and no Session × Genotype interaction *[F(13,221)<1, N.S.]* (Figure 4.6.D), indicating that all subjects showed learning (as indicated by error reduction). However, the main effect of Genotype suggests that the *Dcdc2*$^{del2/del2}$ group maintained a deficit (despite learning) relative to WTs. Next, examination of *working memory incorrect* errors (repeated measures ANOVA across sessions) indicated no differences in errors made between *Dcdc2*$^{del2/del2}$ and WT subjects *[F(1,17)=2.94, N.S.]* (Figure 4.6.E). There was a significant main effect of Session *[F(13,221)=2.66, P<0.01]* indicating that all subjects significantly reduced the number of working memory incorrect errors over time.

Finally, a univariate ANOVA examining the percent frequency of successive angle entries averaged across sessions revealed that *Dcdc2*$^{del2/del2}$ had a greater preference for 45° (adjacent) arm entries in comparison to WT *[F(1,17)=5, P<0.05]* (Figure 4.7.A), and paired samples analysis within the *Dcdc2*$^{del2/del2}$ group indicated that *Dcdc2*$^{del2/del2}$ subjects made significantly more 45° arm entries than arm angles greater than 90° *[t(7)=3.91, P<0.01]*. However analysis of average turn angle for each session showed no main effect of Genotype *[F(1,17)=0.55, N.S.]* (Figure 4.7.b). Furthermore, there was no main effect of Test Session *[F(13,221)=1.38, N.S]* (Figure 4.7.B). This suggests that despite *Dcdc2*$^{del2/del2}$ making more adjacent arm choices, they still utilized larger turn angles in their search strategy so that their average turn angle per test session was similar to WTs.

4.4.3 Sensorimotor ability unaffected by Dcdc2 deletion

A repeated measures ANOVA examining average latency on the rotarod across 5 days of testing found no main effect of Genotype *[F(1,18)=2.47, N.S.]* (Figure 4.8), nor a main effect of Session for either WT *[F(4,40)=1.11, N.S.]* and *Dcdc2*$^{del2/del2}$ *[F(4,32)=0.972, N.S.]*. These results indicate that WT and *Dcdc2*$^{del2/del2}$ subjects had comparable sensorimotor ability and motor learning for this task.

4.4.4 Dcdc2 mutation affects tactile discrimination

Overall analysis of percent interaction with either the 80 grit or 180 grit textures revealed no significant difference of Genotype using a one way ANOVA *[F(1,18)=2.36, N.S.; F(1,18)=2.36, N.S.*, 80 and 180 grit, respectively]. However, individual within-genotype analysis using a paired samples t-test comparing percent interaction between the 80 and 180 grit textures showed that WT subjects spent significantly more time interacting with the 180 (novel) grade texture *[t(10)=-2.92, P<0.05]* as compared to the 80 (familiar) grade texture (Figure 4.9.A). Conversely, *Dcdc2*$^{del2/del2}$ subjects did *not* spend significantly more time with the novel 180 grade texture in comparison to the familiar 80 grade texture. These results suggest that WT mice were able to discriminate between the two different textures, as suggested by the increased percent interaction with the novel texture, while *Dcdc2*$^{del2/del2}$ were unable to perceive a tactile difference.

Further testing was conducted to determine whether differences in tactile discrimination seen in WTs could be attributed to visual cues (see above). Here, paired samples t-tests showed that both WT and *Dcdc2*$^{del2/del2}$ subjects spent a comparable amount of time between the two stimuli *[t(10)=0.12, N.S; t(8)=1.01, N.S*; WT and

Dcdc2$^{del2/del2}$, respectively] (Figure 4.9.B), supporting the view that genotype differences observed in the tactile discrimination task did not reflect WT reliance on visual input.

4.5 Discussion

Current results provide the first evidence for behavioral effects from manipulations of *Dcdc2* in rodents, as evidenced across domains of auditory processing, somatosensory processing, and working and reference memory ability. Specific auditory results showed poor overall performance on the initial Silent Gap task, with no significant Genotype difference. On the subsequent EBT 0–100 ms task, we observed better overall performance, with a Genotype × Duration interaction (*Dcdc2*$^{del2/del2}$ worse than WT at 20ms, 75 ms and 100 ms). Finally, we administered a 0–10 ms EBT task, and here we found a main effect of Genotype (*Dcdc2*$^{del2/del2}$ worse than WT). These combined findings likely reflect co-occurring influences of altered task difficulty together with ongoing experience. However, it is nonetheless interesting that strong *Dcdc2*$^{del2/del2}$ deficits emerged primarily on the shortest-duration version of the task, and this finding appears consistent with literature showing acoustic processing deficits specific to short stimulus durations (i.e., RAP) in language-impaired populations (Benasich & Tallal, 2002; Choudhury et al., 2007; Tallal, 1980). Alternately, results may reflect enhanced effects of prior experience in WT mice, which would suggest a failure of beneficial experience to improve acoustic acuity in *Dcdc2*$^{del2/del2}$ mice. Future studies could parse these different interpretations by administering counter-balanced task batteries in subsets of both *Dcdc2*$^{del2/del2}$ and WT mice.

Persistent memory impairments were also observed in mice with a mutation of *Dcdc2*. Across 14 consecutive days of testing on the 4/8 radial arm water maze, *Dcdc2*$^{del2/del2}$ subjects consistently made more working and reference memory errors. Lack of a motor learning impairment in *Dcdc2*$^{del2/del2}$ subjects suggests that *Dcdc2* function is not associated with gross sensorimotor learning (at least on the rotarod task), thus supporting the view that behavioral deficiencies observed on the 4/8 spatial water maze task were not confounded by underlying sensorimotor deficits. Analysis of turn angles to examine maze solving strategy indicated that *Dcdc2*$^{del2/del2}$ subjects more frequently utilized 45° turns (adjacent arm entries) in their search strategy, however, overall average turn angle within and across test sessions were similar between *Dcdc2*$^{del2/del2}$ and WT subjects. Although both *Dcdc2*$^{del2/del2}$ and WT subjects were capable of learning the water maze task (as evidenced by the significant reduction in total errors), this dichotomy likely reflects the fact that *Dcdc2*$^{del2/del2}$ were less able to recall and identify remaining baited goal arms, and therefore employed a next-arm search strategy, whereas WTs were more likely to utilize extra maze cues to more efficiently navigate the maze. In addition, as the cognitive load of the task increased within a test session (as number of remaining escape platforms decreased), *Dcdc2*$^{del2/del2}$ subjects made considerably more errors suggesting that *Dcdc2*$^{del2/del2}$ had greater difficulty recalling the locations of remaining goal locations.

Previous research has shown that the hippocampus is required to acquire both spatial working and reference memory on different maze tasks, including the 4/8 radial arm maze (Jarrard, 1978; Nadel and MacDonald, 1980; Olton and Papas, 1979). Therefore, it is interesting that impairments in water maze performance were observed

despite absence of gross hippocampal anomalies in *Dcdc2*$^{del2/del2}$ mice (Wang et al., 2011). However, it remains unknown whether *Dcdc2* may directly mediate hippocampal function and circuitry, which may ultimately disrupt memory consolidation. It could also be that abnormalities in other areas relevant to working and reference memory ability (e.g. prefrontal cortex and parietal cortex, respectively) could also contribute to the poor performance observed in mice with a mutation of *Dcdc2*, since *Dcdc2*$^{del2/del2}$ subjects were capable in reducing total errors made across test sessions, suggesting that at least some aspects of task acquisition were retained (Goldman-Rakic, 1995; Soblosky et al., 1996).

Also consistent with our findings is a recent report showing that deletion of the *Dcdc2* gene in mice degrades neural spike timing (Che, Girgenti & LoTurco, 2013), a neuronal parameter likely critical to efficient population coding of rapidly occurring changes in sensory stimuli. Specifically, our results indicate that on the behavioral level mice with *Dcdc2* deletion have difficulty encoding rapid sequential sensory information in both auditory and tactile modalities, the latter reflecting the encoding of frequency of vibrissa contact with sand particles (i.e., vibrissa moving at a fixed rate will contact high frequency gradients at shorter intervals). Our concurrent results also indicate (at the behavioral level) that mice with *Dcdc2* deletion have difficulty encoding rapid sequential sensory information within the auditory domain. In addition, memory disruptions observed could reflect direct alterations in the neural circuitry subserving memory, for example in the accurate encoding of initial sensory input critical to effective memory formation. In fact, GENSAT images indicate high *Dcdc2* promoter activity in layer 4 neurons of sensory cortices (particularly somatosensory, visual, and auditory cortex), as

well as moderate expression in ventral hippocampus (an area of the brain implicated in spatial working memory; Brady, Saul & Wiest, 2010; Gong et al., 2003; Tseng et al., 2008). Currently, the mechanistic relationship between *Dcdc2* and the manifestation of the altered spike timing phenotype remains unclear, but evidence shows that changes in synaptic transmission observed in *Dcdc2*$^{del2/del2}$ subjects are driven by elevated NMDA receptor activity (Che, Girgenti & LoTurco, 2013). Interestingly, aberrant NMDA receptor activity has widely been implicated in impaired memory performance, specifically in working memory ability (Korotkova et al., 2010; Wang et al., 2013).

The current findings must also be taken in the context of evidence using RNAi in rats (Burbridge et al., 2008). For example, other reports have documented RAP deficits associated with disruptions of both *Kiaa0319* and *Dyx1c1* (rodent homologs of candidate dyslexia susceptibility genes), as well as working memory impairments associated with disruption of *Dyx1c1* (Szalkowski et al., 2013; Szalkowski et al., 2011; Szalkowski et al., 2012). Rodent models have also found a correlation between cortical spike timing response patterns to human speech stimuli and behavioral discrimination on an operant conditioning style task (Centanni, Engineer & Kilgard, 2013). Interestingly, RNAi of *Kiaa0319* in auditory cortex has been shown to cause unstable neural representation of human speech sounds in primary auditory cortex using this paradigm (Centanni et al., 2013), a finding consistent with impaired auditory gap detection in *Kiaa0319* knockdown rats as noted above (Szalkowski et al., 2012).

Overall, our current findings correspond to clinical evidence from fMRI and ERP data that suggest impaired auditory temporal processing and/or memory encoding co-occurs with dysfunction in language related neural networks and associated

microcircuitry—all of which may contribute to difficulties in speech perception, language learning, and reading ability (Beneventi et al., 2010a; Beneventi et al., 2010b; Gou, Choudhury & Benasich, 2011; Hornickel & Kraus, 2013; Lehongre et al., 2011; McAnally & Stein, 1996; Raschle et al., 2013; Schulte-Körne et al., 2001; Temple et al., 2000). Alterations in cortical morphology and neocortical activity associated with reading and language related areas are in fact seen in dyslexic individuals with genetic variants of dyslexia risk gene *DCDC2* and/or *KIAA0319* (Cope et al., 2012; Darki et al., 2012; Meda et al., 2008; Pinel et al., 2012).

In conclusion, the current findings taken together with previous clinical and rodent research suggest that *Dcdc2* function may mediate various basic aspects of neurobiological function and behavior including synaptic firing, auditory processing, and memory ability. Although our understanding of the molecular function of *Dcdc2* is still under investigation, concurrent electrophysiological and RNA expression data offer clues to the potential relationships between *Dcdc2,* altered neural activity, and behavioral outcomes. Overall, these findings offer potential targets for both future behavioral and biological research to further understand *DCDC2* function, as well as providing an opportunity to study and better understand the genetic and biological substrates that contribute to disruptions of language and reading dysfunction in humans.

Acknowledgments

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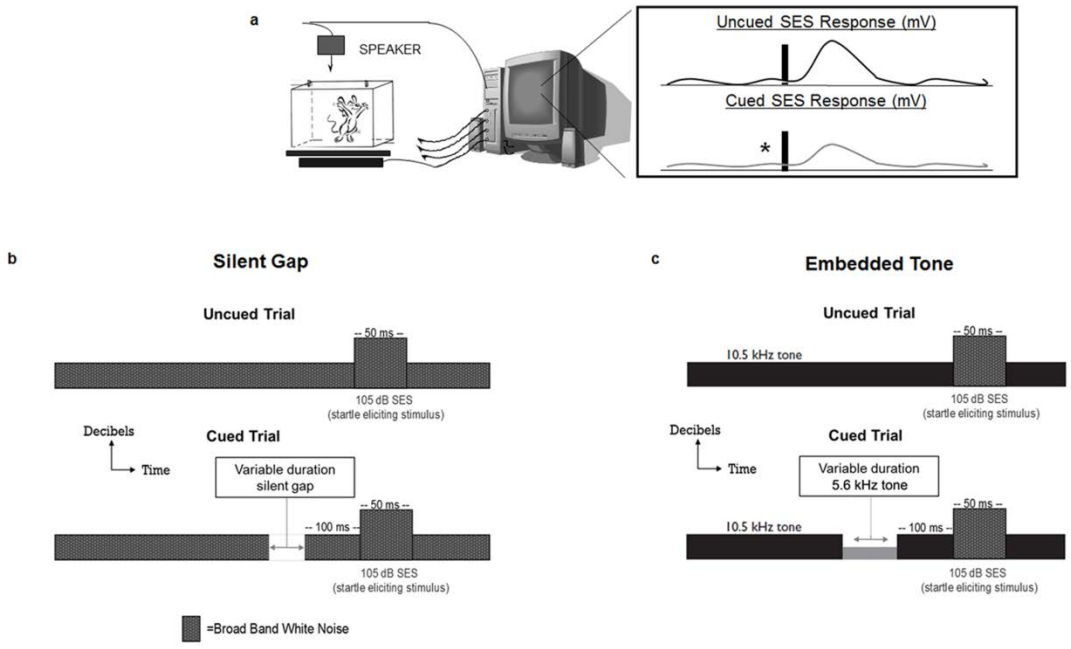

Figure 4.1. Auditory processing assessments. (a) Auditory processing in mice was examined using a modified pre-pulse inhibition (PPI) paradigm. A reduction in the acoustic startle reflex (ASR) to a startle eliciting stimulus (SES) is expected if a cue is presented prior to the SES. A reduction in the ASR during cued trials suggests auditory discrimination. Silent gap (b) examines the subjects' ability to detect discontinuity (varying in duration from 2–100 ms) in a continuous broadband white noise background. The embedded tone (c) task assesses the subjects' ability to detect a variable duration (2–100 ms for long, 2–10 ms for short) 5.6 kHz tone embedded within a constant 10.5 kHz pure tone.

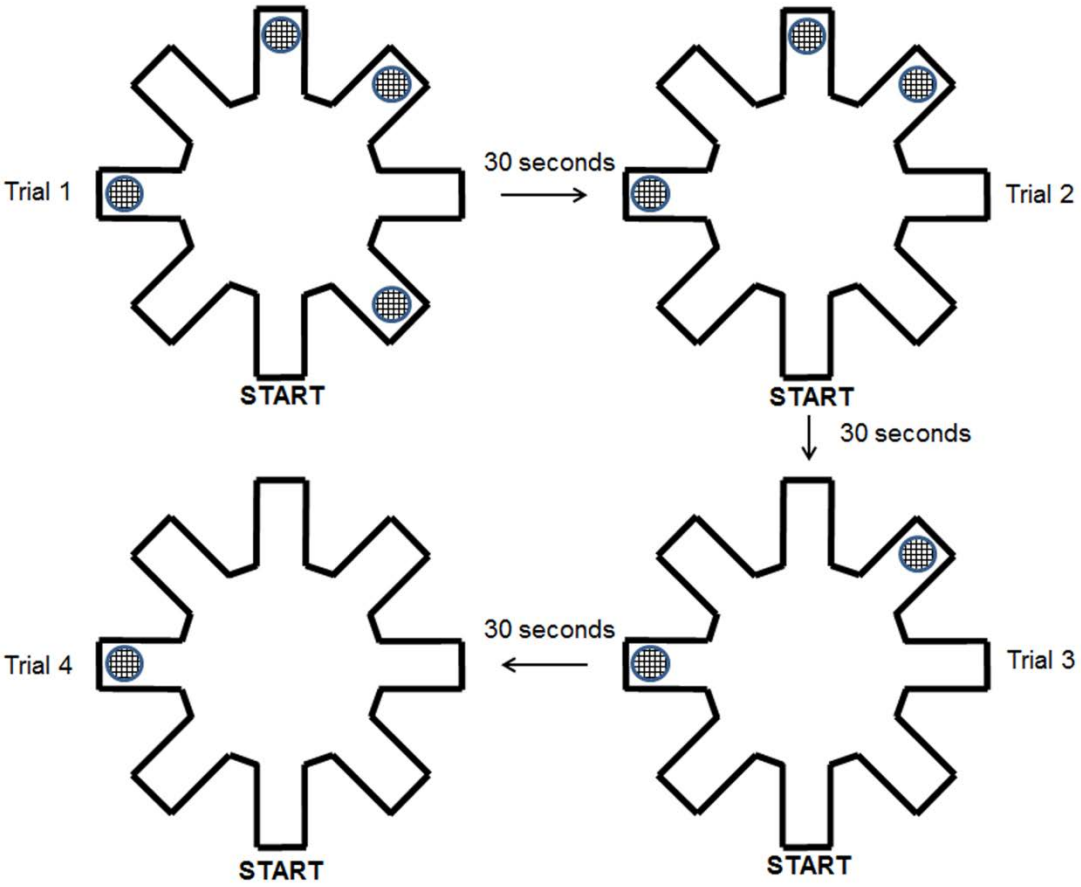

Figure 4.2. Schematic of the 4/8 radial arm water maze and general testing protocol utilized in the experiment to assess both working and reference memory performance. As hidden goal platforms were found by the subject, the recently located goal was removed from the maze prior to the subsequent trial until all 4 platforms were found.

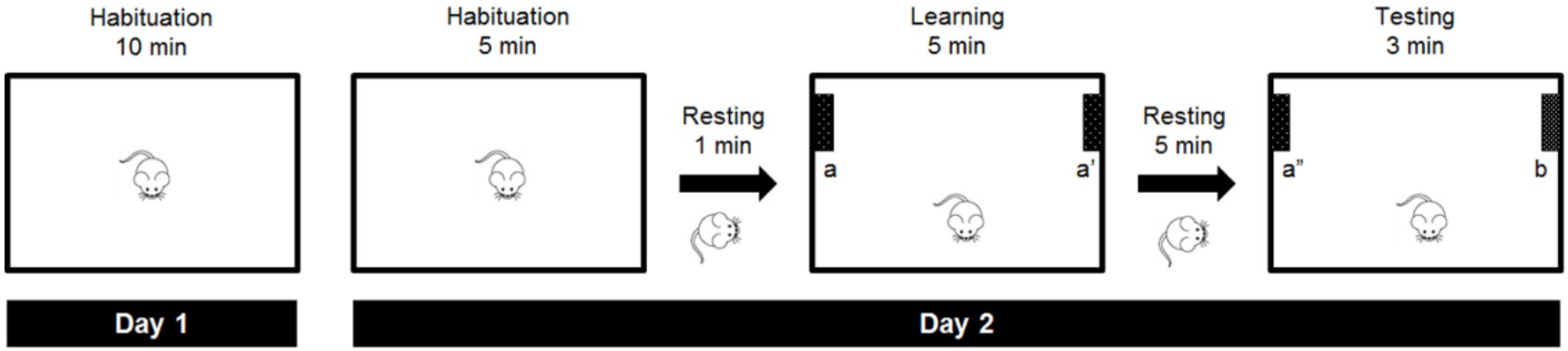

Figure 4.3. Schematic of tactile discrimination task with protocol adapted from Wu et al., 2013. Subjects were examined for their ability to discriminate between a familiar 80 grade sandpaper (a and a' during learning; a" during testing) and a novel 180 grade sandpaper (b).

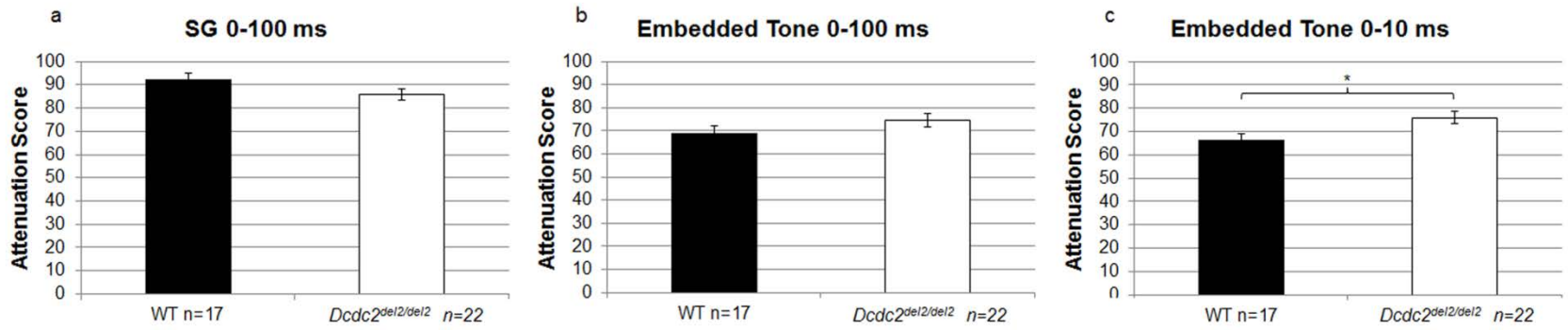

Figure 4.4. *Dcdc2*$^{del2/del2}$ subjects show impaired embedded tone performance. (a) rapid auditory processing performance on the silent gap 0–100 ms paradigm were comparable between *Dcdc2*$^{del2/del2}$ subjects and WT controls. (b) Analysis of embedded tone performance on the 0–100 ms paradigm also saw no significant difference in auditory processing ability, but (c) *Dcdc2*$^{del2/del2}$ subjects did show impaired auditory processing performance on the embedded tone 0–10 ms task. Note that higher attenuation scores indicate poorer auditory discrimination of the cue. *P<0.05.

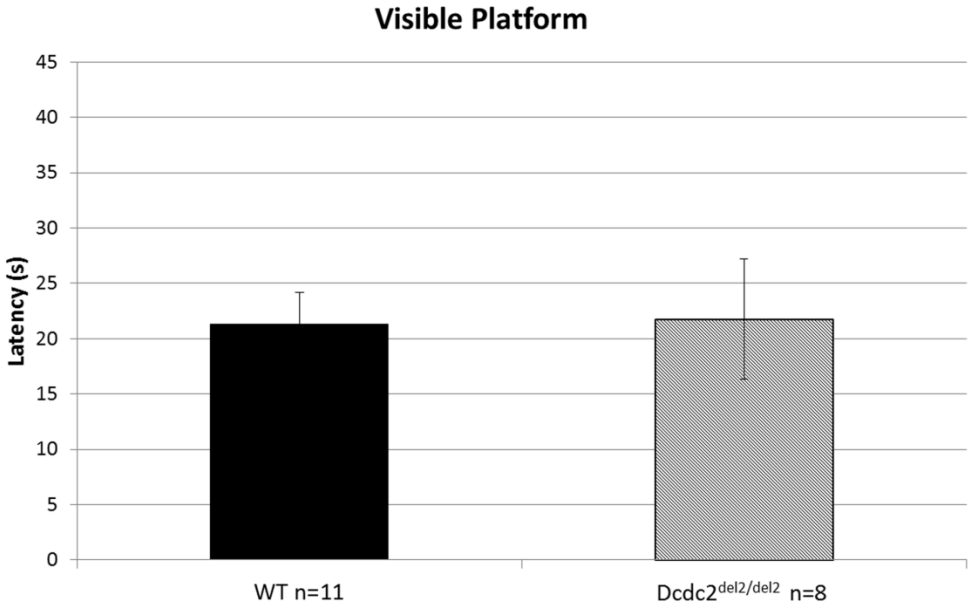

Figure 4.5. Visible platform performance in *Dcdc2*$^{del2/del2}$ and WT mice. *Dcdc2*$^{del2/del2}$ mice were comparable to WT mice on the visible platform water maze task, indicating that all subjects had comparable swimming and visual ability.

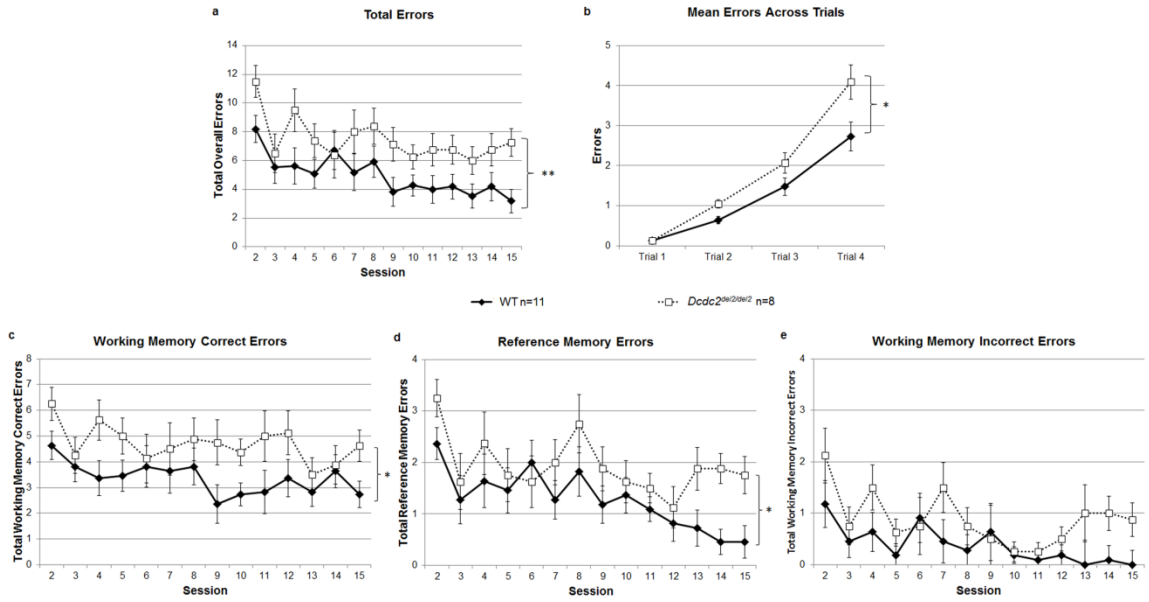

Figure 4.6. General memory impairment in mice with a mutation of *Dcdc2*. (a) Analysis of total errors (working correct, reference, and working incorrect) made across 14 test sessions indicate that *Dcdc2*$^{del2/del2}$ mice made significantly more errors, overall, across all 14 test sessions. (b) Within test session analysis of total errors across trials reveal that *Dcdc2*$^{del2/del2}$ subjects made significantly more errors in the maze across trials than wild type controls. Specifically, as the cognitive load of the task increased (number of remaining escape platforms decreases). (c) Further examination by error type found that *Dcdc2*$^{del2/del2}$ mice made significantly more working memory correct (c) and reference memory (d) errors in comparison to WT mice, but were comparable in the amount of working memory incorrect errors performed (e). *P<0.05; **P<0.01

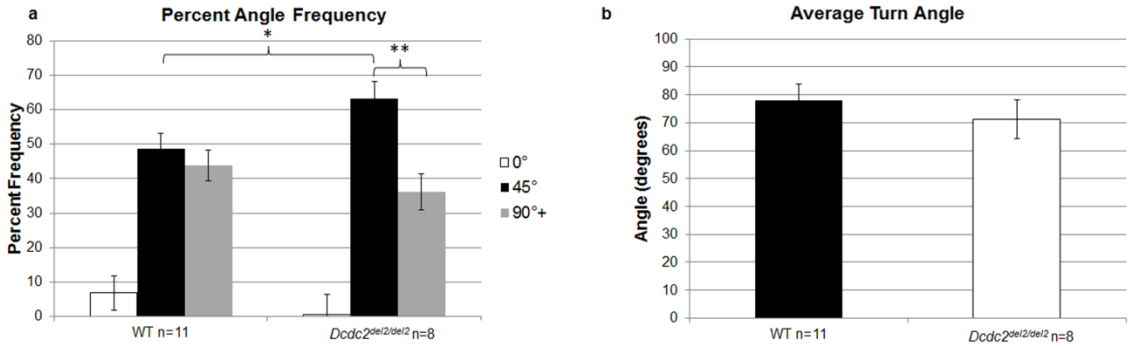

Figure 4.7. Strategy analysis on the 4/8 radial arm maze. (a) *Dcdc2*$^{del2/del2}$ mice showed a greater preference for making 45° (adjacent) arm entries in comparison to WT. Further within subject analysis of *Dcdc2*$^{del2/del2}$ turn angles also revealed that subjects made significantly more 45° turns, overall, than turns 90° or more. (b) However, examination of average turn angle indicate that both *Dcdc2*$^{del2/del2}$ and WT mice had comparable mean turn angles, suggesting that *Dcdc2*$^{del2/del2}$ also had to make a number of larger turn angles greater than 90° together with the increased incidence of 45° turn angles. *P<0.05; **P<0.01

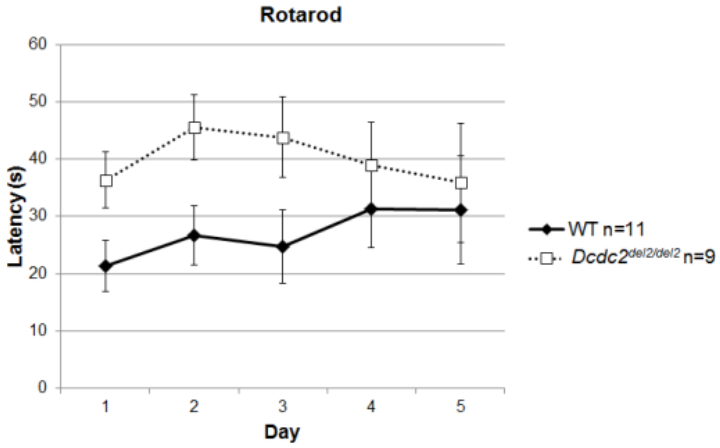

Figure 4.8. Sensorimotor ability in *Dcdc2*$^{del2/del2}$ mice. No differences in sensorimotor performance between *Dcdc2*$^{del2/del2}$ and WT subjects were observed on the rotarod task. Both groups were comparable in their latency to remain on the rotating cylinder across five days of testing.

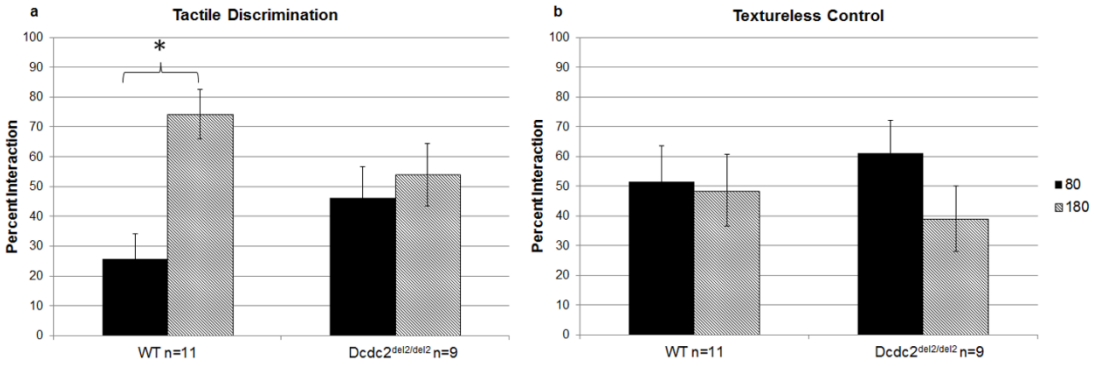

Figure 4.9 *Dcdc2*$^{del2/del2}$ mice show impairments in tactile discrimination in a somatosensory adaptation of the novel object recognition task. (a) WT mice spent significantly more time with the novel 180 grade sandpaper than the familiar 80 grade sandpaper during the testing phase. WT 80 versus WT 180: **P*<0.05. However, *Dcdc2*$^{del2/del2}$ spent a comparable amount of time with both the familiar 80 grade and novel 180 grade sandpaper. (b) A textureless control procedure indicated that both WT and *Dcdc2*$^{del2/del2}$ mice did not use visual information in order to perform the task with both groups spending comparable amounts of time with the textureless "familiar" 80 grade and textureless "novel" 180 grade sandpaper.

CHAPTER 5

Auditory processing anomalies in *Cntnap2* mutant mice

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5.1 Abstract

Autism spectrum disorder (ASD) is characterized by cognitive/behavioral abnormalities that include atypical language, but neurogenetic mechanisms remain poorly understood. Some evidence suggests that aberrant auditory processing—including impaired spectro-temporal processing (e.g., speech in noise, gap detection) and also enhanced spectral perception (e.g., superior pitch discrimination)—may *both* contribute to the anomalous language phenotype. Genetic linkage and association studies have implicated a role for *CNTNAP2* in ASD, and also developmental language disorders. To further examine this inter-relationship, we assessed *Cntnap2* knockout (KO) mice on auditory processing tasks including silent gap detection and pitch discrimination. KO mice showed *deficits* in silent gap detection but *superior* pitch-related discrimination compared to wild types. Findings are consistent with a central role for *CNTNAP2* in the ontogeny and function of neural systems subserving auditory processing, and suggest that disruption of these neural systems could contribute to the atypical language phenotype in ASD.

5.2 Introduction

Autism spectrum disorder (ASD) is a complex neurobehavioral developmental disorder characterized by core anomalies in three behavioral domains, including: 1) social behavior; 2) language development; and 3) repetitive behaviors and restricted interests. Many findings support a genetic basis for ASD, with hundreds of genes implicated, highlighting the phenotypic complexity and heterogeneity of ASD, as well as the need to use intermediate endophenotypes to dissociate the genetic etiology(ies) (Abrahams & Geschwind, 2008). This directive is further exemplified in the call to map basic dimensions of functioning onto genetic and other levels of analysis in NIH's Research Domain Criteria (RDoC) project. Accordingly, the current study reveals a strong link between the *Cntnap2* gene in mice and an atypical auditory processing phenotype highly similar to that observed in ASD. This phenotype is purported to contribute (in the ASD population) to atypical language.

An early study that mapped gene variants of ASD onto a language endophenotype suggested that *CNTNAP2* (contactin-associated protein-like 2) affected language onset and delay (Alarcón et al., 2008). Ongoing data from family linkage and case-control studies also link *CNTNAP2* with ASD, as well as more specific language-related endophenotypes (e.g., nonword repetition; Rodenas-Cuadrado, Ho & Vernes, 2013). Related findings implicate *CNTNAP2* in specific language impairment (SLI; Vernes et al., 2008), as well as language ability in the general population (Whitehouse et al., 2011). Developmentally, *CNTNAP2* appears to modulate brain morphology in cortical regions relevant to ASD-symptomatology (Scott-Van Zeeland et al., 2010). Also, *CNTNAP2* is related to the neurexin family of transmembrane proteins that mediate presynaptic

development, and these proteins are highly expressed in neural language circuitry, and are further associated with ASD (Sudhof, 2008). Interestingly, *CNTNAP2* transcription is downregulated by *FOXP2*, a gene also implicated in language disability (motor planning for vocal learning, verbal dyspraxia; Vernes et al., 2008), although not autism (Newbury et al., 2002).

Animal models have expanded on the above clinical evidence of associations between *CNTNAP2* and language dysfunction. For example, mouse pups with a genetic knockout (KO) of *Cntnap2* (rodent homolog of human *CNTNAP2*) showed reduced ultrasonic vocalizations (Peñagarikano et al., 2011). Studies of *Cntnap2* expression in mice and male songbirds also indicate that *Cntnap2* mediates striatal and cortico-cerebellar circuitry necessary for vocal learning (Condro & White, 2014). However, the specific role of *CNTNAP2* in the development of auditory processing—a function crucial to both vocal learning in animals, and language learning in humans—remains unclear (Brainard & Doupe, 2000; O'Connor, 2012).

Fortuitously, auditory processing is highly amenable to study in rodent models (Fitch et al., 2008b). To address the role of *Cntnap2* in acoustic processing skills fundamental to social communication in humans, we assessed *Cntnap2* KO and wild type (WT) control (C57BL/6J) mice on auditory processing paradigms designed specifically to capture language-relevant endophenotypes. Given the pattern of dichotomous auditory processing behaviors observed in ASD populations from enhanced perception of pitch changes to deficits in more complex spectrotemporal processing, we predict that *Cntnap2* KO mice will show similar superiority in pitch discrimination but impairments in rapid auditory processing (RAP) on a silent gap detection task.

5.3 Materials and Methods

5.3.1 Subjects

22 *Cntnap2* KO mice (B6.129(Cg)-*Cntnap2*^{tm1Pele}*/J*; stock number 017482) and 23 wild type (WT) controls (C57BL/6J; stock number 000664) were obtained from The Jackson Laboratory (Bar Harbor, ME). Subjects were delivered to the University of Connecticut, Department of Psychology in two separate cohorts with Cohort 1 and 2 both arriving at 7 weeks of age (Cohort 1: 12 *Cntnap2* KO, 12 WT; Cohort 2: 10 *Cntnap2* KO, 11 WT). Upon arrival, all subjects were single housed in standard plexiglas laboratory cages (12:12 light/dark cycle) with food and water available *ad lib*. Only male subjects were used for testing based on evidence of a higher incidence of ASD and developmental language impairments in males compared to females (Baron-Cohen et al., 2011). Behavioral testing began at 10–14 weeks of age, and occurred during the subjects' light cycle. Order of auditory task presentation was counterbalanced within each cohort to control for potential experience effects on subsequent auditory testing. Cohort 1 was assessed on the silent gap and embedded tone paradigms only, while Cohort 2 was assessed on silent gap, embedded tone, and pitch discrimination (as reflected in a lower n for pitch discrimination results). All procedures were performed blind to subject genotype and were conducted in compliance with the National Institutes of Health and approved by the University of Connecticut's Institutional Animal Care and Use Committee (IACUC).

5.3.2 Acoustic Startle reduction paradigm

Auditory processing was assessed using a modified pre-pulse inhibition (PPI) paradigm. In brief, subjects were observed for their ability to reduce their acoustic startle reflex (ASR; a rapid, involuntary, contraction of muscles elicited by an unexpected intense

noise burst) in response to an auditory cue (pre-pulse) presented 50–500 ms prior to the loud noise burst (startle eliciting stimulus; SES; see Fitch et al., 2008b, for review). For all auditory processing procedures, subjects were placed on individual load-cell platforms (PHM-250, MED Associates, St. Albans, VT) and exposed to various auditory stimuli generated using a Dell Pentium D PC with custom programs (executed via RPvdsEx and RX6 multifunction processor, Tucker Davis Technologies, Alachua, FL). Auditory stimuli were amplified using a Niles SI-1260 Integration Amplifier (Niles Audio Corp., Carlsbad, CA) and delivered through powered Yamaha YHT-M100 speakers (Buena Park, CA). The subject's ASR was measured using the output voltage from the load-cell platform, sent through a linear amplifier (PHM-250U; Med Associates, St Albans, VT) into a Biopac MP150 acquisition system (Biopac Systems, Goleta, CA) and recorded on an iMac 7.1 running Acknowledge 4.1. For data analysis, the maximum peak output value of the ASR was extracted from a 200 ms window immediately following the onset of the SES. The maximum peak startle amplitude within each designated epoch was coded for cued and uncued trials. For all auditory paradigms, the SES was a 50 ms, 105 dB, white noise burst. During cued trials, the auditory cue was presented 100 ms prior to the SES. If the subject was able to detect the cue, then attenuation (reduction) of the ASR was expected relative to the ASR elicited when the cue was not present (or could not be detected). Reduction of the ASR was quantified and analyzed using an "attenuation score" (ATT), specifically comparing the mean cued ASR to the mean uncued ASR, multiplied by 100 ([average cued ASR/average uncued ASR]*100). Exploitation of the startle reduction paradigm allowed for manipulation of the auditory cue in varying

temporal and psychophysical gradations in order to determine a “threshold” of auditory discrimination across various auditory stimuli.

5.3.3 Normal Single Tone (NST)

Prior to auditory testing, all subjects were assessed on NST task to rule out any underlying impairments that could affect performance on subsequent auditory testing (e.g. hearing impairment, dysfunctional startle gating mechanism). This task also provided an index of baseline auditory pre-pulse inhibition ability across test groups. For this task, subjects had to detect a simple single tone (50 ms, 75 dB, 8,000 Hz tone) presented 50 ms prior to the 50 ms, 105 dB, SES in a silent background. The NST task included 104 cued and uncued (no tone prior to SES) trials presented pseudorandomly throughout the test session with a variable inter-trial interval (ITI) ranging from 16–24 s.

5.3.4 Silent Gap 0–100 ms

The Silent Gap (SG) task assessed the ability to detect breaks (silent gaps) within a continuous broadband white noise background. A test session consisted of 300 consecutive trials with varying inter-trial intervals ranging from 16–24 s. All trials employed a continuous 75 dB broadband white noise background with cued and uncued trials pseudorandomly presented throughout the test session. During cued trials, a silent gap of variable duration (2, 5, 10, 20, 30, 40, 50, 75, 100 ms) was presented with a 2 ms up/down dB ramp, 100 ms prior to the 50 ms, 105 dB, white noise burst with gap conditions also pseudorandomly ordered throughout the test session. Uncued trials were a 0 ms gap condition (no silent gap) prior to the SES. For this task, subjects were given one test session a day across five consecutive test days. Silent gap testing was conducted in both Cohorts 1 and 2.

5.3.5 Variable duration embedded tone

A test session of the variable duration embedded tone task consisted of 300 sequential trials with varying inter-trial intervals ranging from 16–24 s. This auditory paradigm examined the subject's ability to detect a change in pure tone frequency from a standard background tone across variable tone durations presented pseudorandomly. For this task, the cue was a 75 dB, 5600 Hz pure tone of variable duration embedded within a standard 75 dB, 10500 Hz continuous background pure tone (2 ms up/down linear frequency ramp). On cued trials, the embedded tone was presented 100 ms prior to the 50 ms, 105 dB, SES. Uncued trials consisted of only the 105000 Hz pure tone background and presentation of the SES. Two types of variable duration embedded tone paradigms were utilized in this study—a long duration embedded tone task which examined embedded tone durations ranging from 0 ms (uncued) up to 100 ms (e.g. 2, 5, 10, 20, 30, 40, 50, 75, 100 ms), and a short duration embedded tone task which assessed durations ranging from 0 ms (uncued) up to 10 ms (2, 3, 4, 5, 6, 7, 8, 9, 10 ms). Subjects were first tested on the long duration embedded tone (0–100 ms) over a span of four consecutive days (one test session per day). Assessment on the short duration embedded tone (0–10 ms) followed the long duration paradigm and took place over the span of five consecutive days (one test session per day). Embedded tone testing (both long and short durations) was performed on Cohorts 1 and 2.

5.3.6 Pitch Discrimination

This task was used to examine the subject's ability to detect very small changes in pitch embedded within a background tone. A test session comprised 300 sequential trials with a variable ITI ranging from16–24 s. The cue was a 300 ms, 75 dB tone of variable

frequency embedded within a standard 75 dB, 10500 Hz background pure tone (2 ms up/down linear frequency ramp), presented 100 ms prior to the SES. The experimental frequencies used for the pitch discrimination task deviated from the standard background frequency by as much as 75 Hz, to as little as 5 Hz—specifically examining cue frequencies of 10425, 10450, 10475, 10490, 10495, 10510, 10515, 10525, and 10550 Hz. Uncued trials did not include a frequency deviant prior to the SES. Only subjects in Cohort 2 were assessed on the pitch discrimination task. Pitch discrimination testing took place across three consecutive days of testing (one test session per day).

5.3.7 Statistical Analysis

Normal Single Tone (baseline control) data was examined using a one-way analysis of variance (ANOVA) comparing *Cntnap2* KO and WT attenuation scores. Due to a small significant difference (although both groups showed robust detection), performance on NST was used as a covariate in subsequent statistical analysis. Experimental paradigms were analyzed using a mixed factorial design. Differences in attenuation (ATT) scores during silent gap performance was examined using a 2 × 5 × 9 repeated measures analysis of variance (ANOVA) with Genotype (2 levels: *Cntnap2* KO and WT) as the between-subjects variable, and Day (5 levels) and Gap duration (9 levels) as the within-subjects variable. For the embedded tone paradigms, a 2 × 4 × 9 and a 2 × 5 × 9 (for long and short duration EBT, respectively) repeated measures ANOVA was used to compare differences in ATT scores. Genotype (2 levels) was the between-subjects measure, with Day (4 and 5 levels respectively for long and short duration EBT) and Tone duration (9 levels) as within-subject measures. Pitch discrimination data was analyzed using a 2 × 3 × 9 repeated measures ANOVA with Genotype as the between measure, and Day (3

levels) and Frequency (9 levels) as the within measures. In addition, a temporal threshold for gap detection, embedded tone detection, and frequency difference for discrimination (pitch discrimination), respectively, were each assessed using a paired samples t-test comparing mean cued and uncued ASR *within* each Genotype, at each Duration. If the raw cued ASR was significantly reduced relative to the uncued ASR at a given cue duration, then auditory discrimination of the auditory cue (e.g. silent gap, embedded tone, small frequency change) was supported.

5.4 Results

5.4.1 Justification to pool Cohorts 1 and 2

Subjects' performances across cohorts were compared for the silent gap and embedded tone (both long duration 0–100 ms and short duration 0–10 ms) paradigms. Repeated measures ANOVA used Genotype and Cohort as the between-subjects measures, and within measures consisted of the variables previously described for the silent gap and embedded tone paradigms (Day, Duration). We found no significant Genotype × Cohort interaction for either task, indicating that performance across silent gap and embedded tone tasks were comparable across cohorts. Therefore, data from the silent gap and embedded tone paradigms were pooled across cohorts for further analysis, rendering a final n=22 for *Cntnap2* KO and n=23 for WT controls.

5.4.2 Normal Single Tone

Cntnap2 KO and wild type control mice were initially tested on a standard single tone task to establish baseline hearing and PPI ability. During cued trials, a 50 ms, 8 kHz tone pip was presented 50 ms prior to the presentation of the SES (Figure 5.1.A). Both *Cntnap2* KO and WT mice showed significant auditory PPI, but baseline auditory PPI in

Cntnap2 KO mice was slightly different from WT (Figure 5.1.B), and thus further analyses included single tone attenuation scores as a covariate.

5.4.3 Silent Gap

Once baseline auditory PPI was established, mice completed a variable-duration silent gap detection task in which a silent gap within continuous white noise served as the PPI cue (Figure 5.2.A). Results showed that *Cntnap2* KO subjects were impaired on silent gap detection compared to WT controls $[F(1,40)=6.29, p<0.05]$ (Figure 5.2.B). Attenuation scores were significantly higher in *Cntnap2* KO mice, indicating *Cntnap2* mutants were not as proficient as WT in detecting short duration silent gaps. WTs had a detection threshold of 5 ms (i.e., could detect gap durations 5 ms and longer) $[t(22)=4.55, p<0.001]$, while *Cntnap2* KOs had a detection threshold of 20 ms $[t(21)=2.42, p<0.05]$. Thus although *Cntnap2* KO mice were capable of silent gap detection, their threshold was significantly higher (a longer duration) than that of WT mice.

5.4.4 Embedded Tone

As in the silent gap task, subjects were exposed to 300 trials in which a 5600 Hz pure tone PPI cue of variable duration was superimposed on a background 10500 Hz pure tone with 2 ms up/down linear frequency ramp (Figure 5.3.A). Both groups showed significant ASR reduction at all cue durations with results indicating that *Cntnap2* KO performance was superior to WT on the embedded tone 0–100 ms $[F(1,40)=9.39, p<0.001]$ and 0–10 ms $[F(1,40)=6.27, p<0.05]$ Figure 5.3.B and Figure 5.3.C). Lower attenuation scores for *Cntnap2* KOs indicate enhanced detection of the 5600 Hz tone, even at short durations.

5.4.5 Pitch Discrimination

For the pitch discrimination task, a variable frequency change from a constant 10500 Hz background tone served as the cue (Fig. 5.4.A). Results again revealed enhanced discrimination of the pitch discrepancy in *Cntnap2* KO mice relative to WT mice *[F(1,18)=5.7, p<0.05]*. Furthermore, a significant Genotype × Frequency interaction indicated that both *Cntnap2* KO and WT subjects showed difficulty in discriminating tone changes closest to the 10500 Hz background (e.g. 9990–10510 Hz) *[F(2,36)=4.27, p<0.05]* (Figure 5.4.B), but as the frequency difference between the background and cue increased (i.e., greater than 10 Hz difference), *Cntnap2* KO showed greater attenuation and thus better discrimination compared to WTs.

5.5 Discussion

Mice with the *Cntnap2* genetic mutation showed a dissociation of auditory processing abilities: on the one hand, impairments in temporal silent gap detection; on the other hand, enhanced frequency discrimination in an embedded tone paradigm. Interestingly when the same temporal parameters of the silent gap task were transferred to a spectral task, the mutants showed performance at levels superior to WT. This finding is particularly striking given that enhanced auditory perceptual abilities have never been observed in our lab in any other mutant rodent model.

Importantly, although both silent gap and embedded tone paradigms examined the temporal processing of auditory stimuli, the silent gap task required subjects to detect a discontinuation (OFF versus ON) in broadband white noise, while the embedded tone task assessed detection of an uninterrupted shift in an ongoing frequency. Human

psychophysical studies of detection thresholds for frequency shifts separated by either a silent gap or a continuous FM sweep do report significantly lower detection thresholds in the continuous FM condition (e.g., better performance), indicating that short changes in two discrete tones are easier to detect when the tones are smoothly connected. Authors suggest this may be due to the perception of a “single auditory object” versus a discontinuous one (Demany, Carlyon & Semal, 2009). The continuous frequency change tested in the embedded tone paradigm might therefore be characterized as processing of a “local” acoustic cue (i.e., identifying details within a whole auditory "object"; (Mottron et al., 2006; Ouimet et al., 2012). In contrast, the silent gap paradigm requires the processing of a temporal envelope with discrete on/off cues presented in rapid succession, thus arguably tapping more “global” spectro-temporally complex features (i.e., integrating components to identify a whole; (Ouimet et al., 2012).

The current findings are consistent with the intriguing pattern of atypical auditory processing behaviors observed in ASD individuals, where perception of "local" auditory features (e.g., pitch, loudness) is enhanced (Bonnel et al., 2010; O'Connor, 2012), but deficits in processing more "global" spectro-temporal information (e.g., gaps in noise, speech in noise) are also seen (Alcántara et al., 2012; O'Connor, 2012; Ouimet et al., 2012). Together, findings are consistent with theories of "weak central coherence" (WCC) or “enhanced perceptual functioning”, which suggest that individuals with ASD have a bias towards local over global informational cues across several modalities (visual, auditory) (Happé & Frith, 2006; Mottron et al., 2006).

In the context of impaired language development in ASD, enhanced auditory perceptual abilities—such as increased sensitivity to pitch—may be associated with

difficulties in generalizing across simple acoustic details to form broader phonemic categories (Eigsti & Fein, 2013). This hypothesis is supported by ERP data showing atypical mismatch negativity responses in auditory cortex to simple tonal stimuli (reflecting advantages in local pitch discrimination in ASD individuals (Lepistö et al., 2005), along with impaired processing of more spectro-temporally complex stimuli such as speech syllable changes (Kuhl et al., 2005). The latter abnormalities may also impede language development in ASD, given that the early ability to discriminate and process rapidly changing and temporally complex stimuli (present in consonant transitions) is a strong predictor of later language development, and that impairments in rapid acoustic processing are implicated in developmental language disorders including dyslexia and SLI (Fitch & Tallal, 2003).

Interestingly, physiologic anomalies in acoustic processing have been documented at a variety of levels of the auditory pathway in ASD individuals. Various studies have revealed abnormal cortical MMN responses to acoustic stimuli ranging from simple tones to speech in noise in ASD populations (Kuhl et al., 2005; Lepistö et al., 2005). Abnormal auditory brainstem responses (ABR) to rapidly presented (broad-spectrum) click stimuli have also been observed in ASD (Roth et al., 2012). This is consistent with physiologic evidence that neural processing of silent gaps embedded in noise occurs at the level of the brainstem and auditory periphery (Phillips et al., 1997), suggesting in turn that some anomalous acoustic processing in ASD *may* occur at relatively low levels of the auditory processing stream. These findings contrast studies of "cross-channel" processing (i.e., processing across frequencies), which are thought to reflect higher order neural processing (e.g., cortical; Phillips et al., 1997), thus suggesting

that frequency-related enhancements in ASD may occur at higher levels of the auditory stream.

The current findings highlight auditory processing abnormalities, including a profile of both impaired and enhanced functioning, associated with genetic variants of *CNTNAP2*, and suggest that these anomalies may in turn contribute to the atypical language phenotype of ASD.

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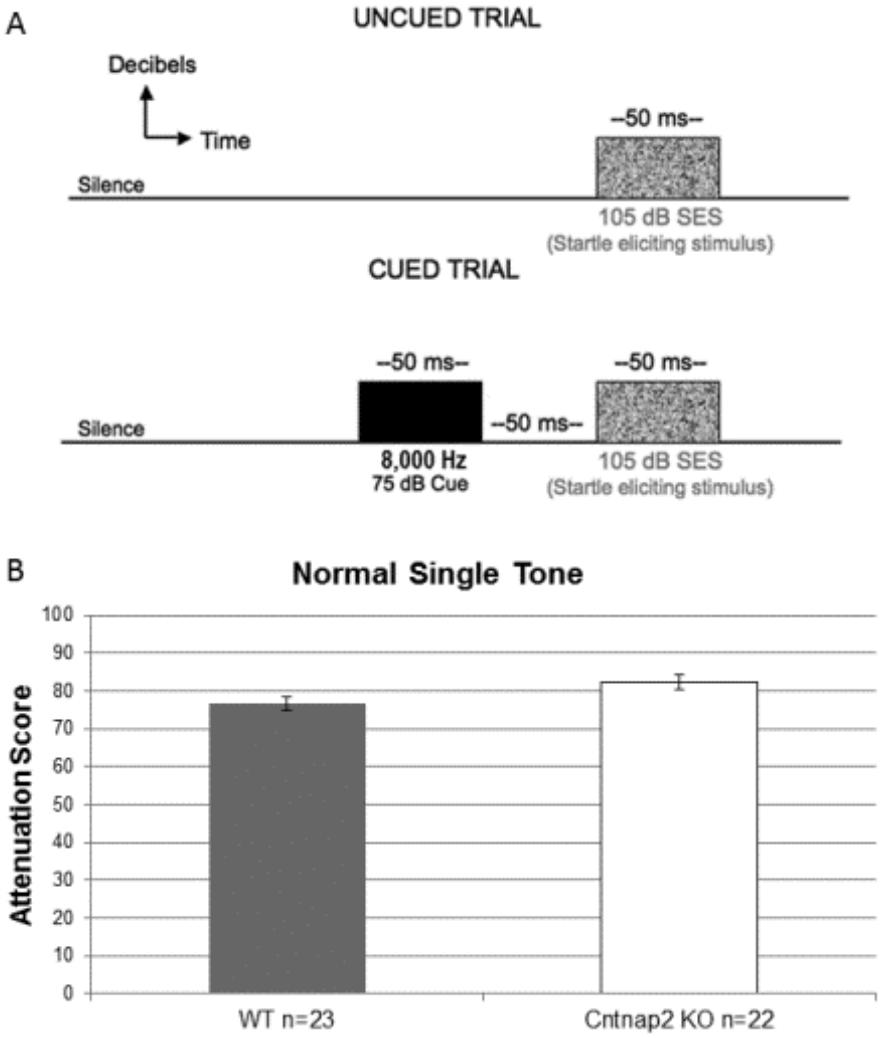

Figure 5.1. Normal single tone control task. (A) Schematic of the normal single tone task, a measure of baseline auditory pre-pulse inhibition ability. The paradigm examines whether subjects are capable of hearing and have an intact startle gating mechanism. (B) *Cntnap2* KO and WT subjects both showed highly significant detection of the NST (with chance levels at 100%), but scores were significantly different and therefore NST scores were used as a within-subject covariate for all statistical analyses.

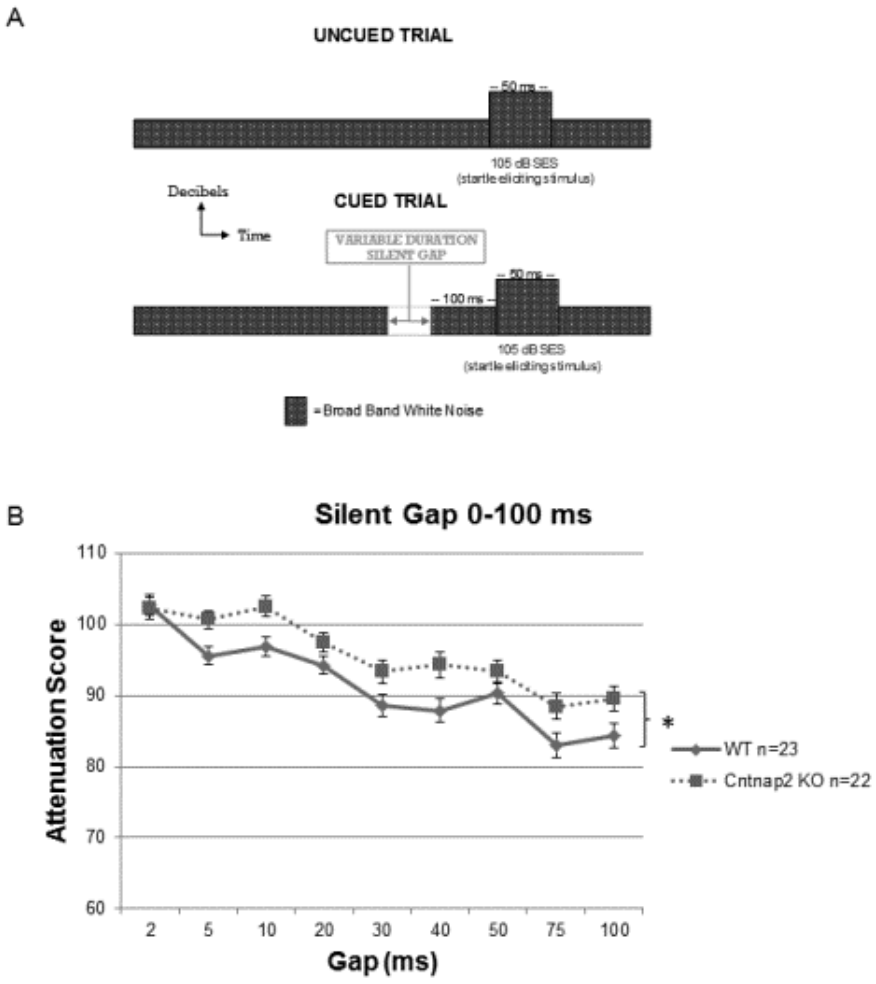

Figure 5.2. Silent Gap 0–100 ms performance of *Cntnap2* KO mice. (A) Overview of the silent gap paradigm. During cued trials, subjects detect silent gaps embedded in broadband white noise. One session (one per day over 5 consecutive days) included 300 trials, with pseudorandom presentation of variable duration silent gaps (2, 5, 10, 20, 30, 40, 50, 75, and 100 ms, with 2 ms up/down dB ramp (except at 2 ms)). (B) *Cntnap2* KO subjects show impaired gap detection compared to WT controls. Attenuation scores were significantly higher in *Cntnap2* KO mice, indicating *Cntnap2* mutants were not as proficient as WT in detecting short duration silent gaps. WTs had a detection threshold of 5 ms; *Cntnap2* KOs had a detection threshold of 20 ms. (*$P<0.05$)

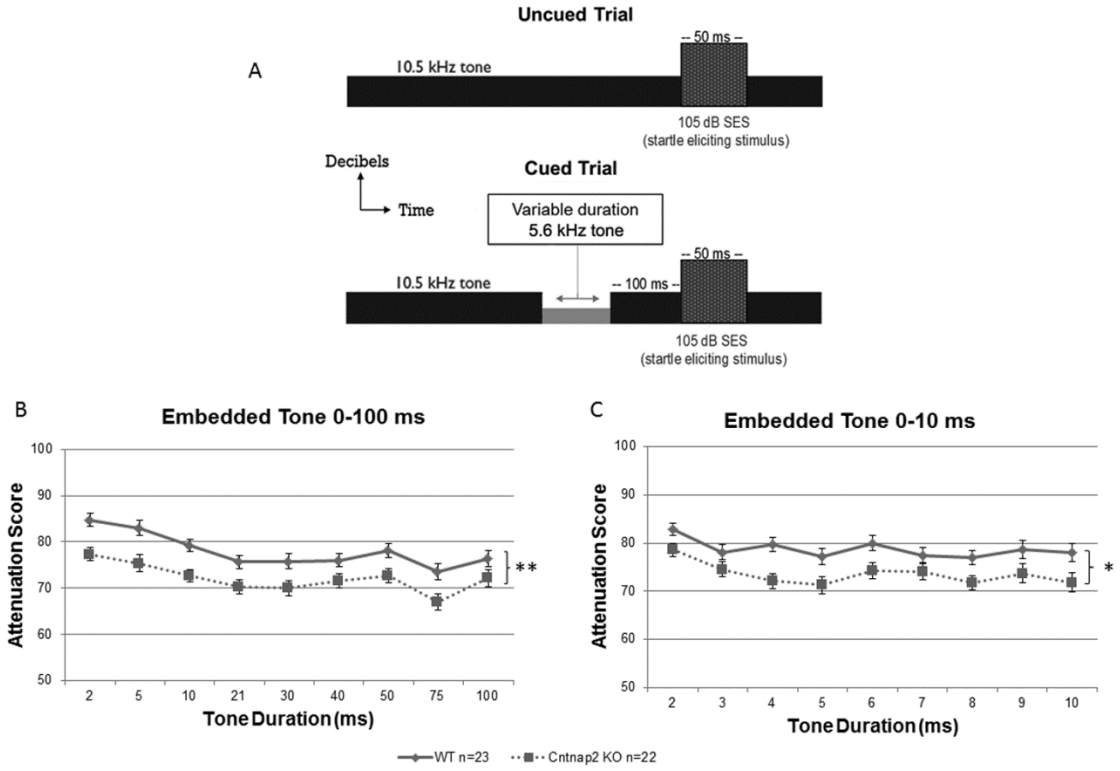

Figure 5.3. Auditory processing behavior on the embedded tone paradigm. (A) General schematic of the embedded tone paradigm, measuring detection of a brief 5600 Hz tone embedded in a 10500 Hz background. Cue durations ranged from 0 ms (uncued) to 100 ms for the long duration task (as in silent gap), and 0 ms to 10 ms for the short duration. Cue durations were pseudorandomly presented, and subjects received one test session per day over four consecutive days (long duration), and five consecutive days (short duration). *Cntnap2* KO performance was superior to WT on the embedded tone 0–100 ms (B), and 0–10 ms (C). Lower attenuation scores for *Cntnap2* KOs indicate enhanced detection of the 5600 Hz tone, even at short durations. (*$P<0.05$; **$P<0.001$)

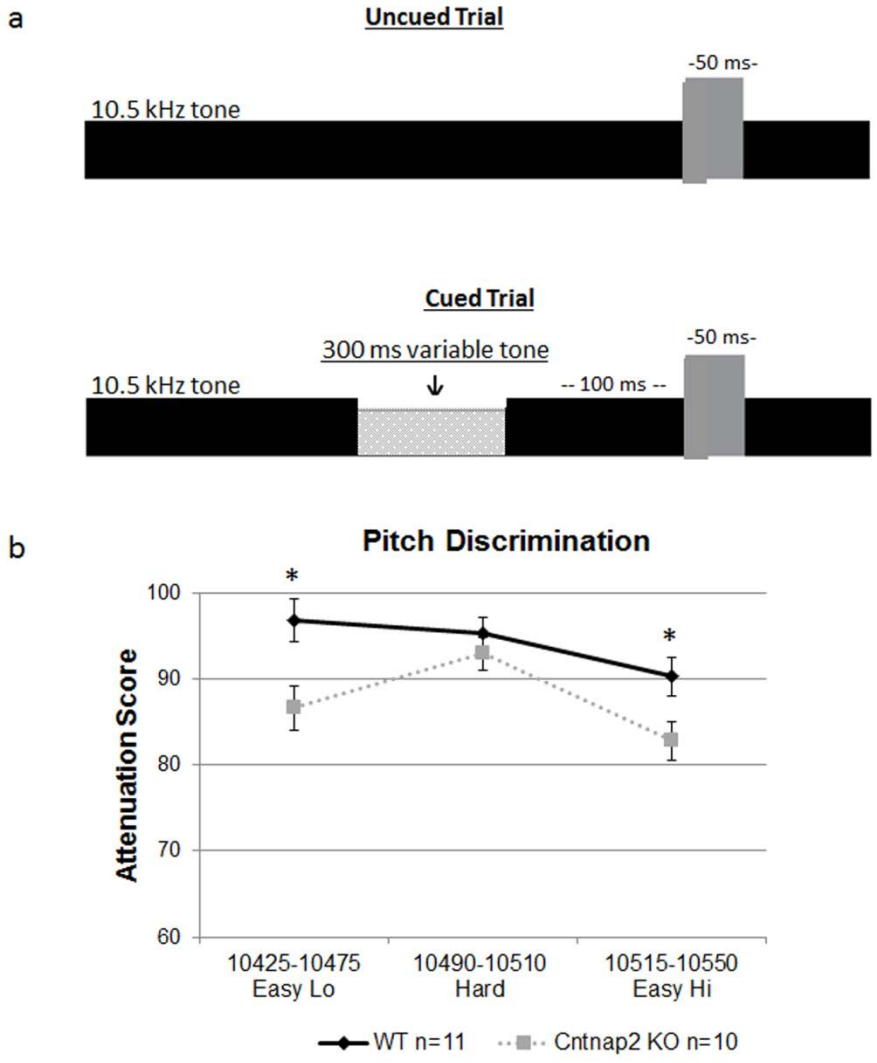

Figure 5.4. Pitch discrimination in *Cntnap2* KO mice. (A) The pitch discrimination task assessed detection of small changes in frequency from a 10500 Hz background tone. Cued trials consisted of a 300 ms frequency change from the standard frequency (delta range 75 Hz to 5 Hz). A test session (one per day for three days) consisted of 300 trials, with different cue frequencies presented pseudorandomly. (B) Examination of small changes in frequency revealed enhanced pitch discrimination performance in *Cntnap2* KO subjects as reflected by significantly lower attenuation scores to WT. A significant Genotype × Frequency interaction indicated WTs and *Cntnap2* KOs were unable to

discriminate frequency changes very close to the standard, but as frequency differences became detectable, *Cntnap2* mutant subjects showed a perceptual enhancement compared to WT. (*$P<0.05$)

CHAPTER 6

Morphological changes in the medial geniculate nucleus of *Cntnap2* KO mice

Unpublished Data

6.1 Abstract

Contactin-associated protein-like2 (*CNTNAP2*) is a gene that has been implicated various genetic epidemiological studies related to general language variation, as well as language disability in autism spectrum disorder (ASD) and specific language impairment (SLI). Investigation of gene-brain-behavior relationships in the context of ASD and SLI have been further facilitated through experimentation with the *Cntnap2* knockout (KO) mouse. Previous examination of *Cntnap2* KO mice in our lab revealed an intriguing pattern of auditory processing behavior, involving both enhanced auditory perception of pitch related information (pitch discrimination), and simultaneous impaired detection of silent gaps embedded within broadband white noise. Based on these findings, the purpose of the current experiment was to examine whether corresponding changes in subcortical cellular morphology and organization along the central auditory pathway could be identified in this mutant. We focused stereologic analysis on the medial geniculate nucleus (MGN; auditory thalamus) and the ventral and dorsal subdivisions of the cochlear nucleus (VCN and DCN), specifically assessing for neuron number and cell size. Results indicated significant differences in MGN morphology, including reductions in neuronal numbers, as well as a shift in the cell size distribution toward smaller cells in *Cntnap2* KO mice. No differences in VCN or DCN of mutants were observed using stereological measures. Current findings support a link between altered MGN

morphology and atypical auditory processing behaviors, as well as elucidating additional neural structural targets affected by the *Cntnap2* mutation in mice.

6.2 Introduction

Contactin-associated protein-like2 (*CNTNAP2*) is a gene of interest in both autism spectrum disorder (ASD) and specific language impairment (SLI), based on evidence from both genetic epidemiological and molecular studies (Alarcón et al., 2008; Arking et al., 2008; Bakkaloglu et al., 2008; Nord et al., 2011; Rodenas-Cuadrado, Ho & Vernes, 2013; Strauss et al., 2006; Vernes et al., 2008). Behaviorally, genetic variants of *CNTNAP2* are associated with language related endophenotypes including age of language onset, non-word repetition, and expressive and receptive language abilities (see Rodenas-Cuadrado, Ho & Vernes, 2013, for review). Biologically, *Cntnap2* is localized in nodes of Ranvier to cluster K+ channels in myelinated neurons (Poliak et al., 1999), and it is of particular interest as a candidate gene for ASD because of its biological relation to the neurexin superfamily—a family of proteins that mediate presynaptic development, and are also associated with ASD (Sudhof, 2008). In a genome-wide analysis of cortical patterning focusing on the perisylvian region in humans—an area of the neocortex highly implicated in language-related processes—it was found that *CNTNAP2* showed enriched expression, suggesting that *CNTNAP2* may play a role in the development and maintenance of cortical patterning and connectivity in language-relevant cortical areas (Abrahams et al., 2007). Additional functional and structural neuroimaging studies also revealed an association between *CNTNAP2* anomalies and alterations in frontal connectivity, as well as region specific reductions in grey and white matter within occipital, temporal, and frontal lobes—regions that participate in frontotemporal-subcortical circuits thought to subserve learning and vocal communication (Alarcón et al., 2008; Scott-Van Zeeland et al., 2010; Tan et al., 2010).

Given this body of knowledge, we (along with others) hypothesized that language-related impairments observed in individuals with potential risk variants of *CNTNAP2* could be mediated by underlying disruptions in neural systems associated with language learning and ability, and moreover that this association might include neural substrates for core functions such as auditory processing.

The thalamus is considered a "relay" station for a majority of sensory inputs coming into the neocortex, with topographic projections to visual, motor, somatosensory, and auditory cortices. In turn, corticofugal projections to the thalamus and other subcortical targets generate complex thalamo-cortical/cortico-thalamic loops, and allow for further modulation of sensory input and processing between the thalamus and neocortex, as well as brainstem. Various lines of evidence using *in vivo* neuroimaging techniques have shown fundamental differences in the thalamus of ASD individuals, including reduced volume (Tamura et al., 2010; Tsatsanis et al., 2003), altered neurochemical composition (Friedman et al., 2003), and abnormal thalamocortical connectivity (Cheon et al., 2011; Chugani et al., 1997; Muller et al., 1998; Mizuno et al., 2006; Nair et al., 2013), suggesting that the thalamus may play a role in the behavioral etiology of ASD symptoms. *CNTNAP2* expression has also been reported in the thalamus of humans, adult mice, and zebrafinch (Alarcón et al., 2008; Lein et al., 2007; Panaitof et al., 2010). However, no functional studies have been conducted to directly examine how differences in genetic variants of *CNTNAP2* may alter thalamic morphology or connectivity with cortex and/or brainstem.

Atypical auditory processing behavior has been reported in ASD individuals, ranging from enhanced perception of simple auditory information such as pitch

identification and discrimination, to deficits in more spectro-temporally complex auditory stimuli such as detecting speech in noise, as well as silent gap detection embedded within noise (see O'Connor, 2012 and Ouimet et al., 2012, for review). Furthermore, electrophysiological examination of ASD individuals also reveals impaired speech and auditory processing, including weaker, delayed, and/or sometimes unreliable neural signals in the cortex (Gandal et al.; Kujala et al., 2007; Lepistö et al., 2005; Lepistö et al., 2006; Roberts et al., 2011). Alterations in neural responses to auditory stimuli also extend to brainstem structures, with data showing a prolonged auditory brainstem response (ABR) in ASD children with language delay in comparison to typically developing controls (Roth et al., 2012). It is thought that abnormalities in low-level auditory processing (as described above) may lead in turn to impaired language ability, which comprises a core component of ASD symptomatology (Eigsti & Fein, 2013). However, it remains unclear how aberrant auditory processing behaviors may manifest within the neural system, and specifically within the central auditory pathway. Given previous research showing structural changes in the overall morphology of the thalamus, changes specific to the auditory nucleus of the thalamus (medial geniculate nucleus; MGN) could help explain the abnormal auditory processing phenotype observed in ASD. Also, given that changes in ABR are observed in ASD, structural disturbances may also be present in brainstem auditory structures within the central auditory pathway (e.g., cochlear nucleus).

Examination of the potential relationship between ASD, auditory processing behaviors, and changes in subcortical structures within the central auditory pathway is necessary, but a relevant model of ASD must be used. Luckily, previous work conducted in our lab revealed a fascinating dissociation of auditory processing behaviors in *Cntnap2*

(rodent homolog of *CNTNAP2*) KO mice that paralleled the co-occurring pattern of enhanced and deficient auditory abilities observed in ASD individuals. Specifically, *Cntnap2* KO mice were impaired on a variable duration silent gap detection task, but showed enhanced pitch discrimination ability in comparison to wild type (C57BL/6J) controls. To investigate whether differences in subcortical anatomy within the central auditory pathway could be related to this abnormal pattern of auditory processing behaviors in *Cntnap2* KO mice, we conducted a stereological assessment of the MGN and the ventral and dorsal subdivisions of the cochlear nucleus (VCN and DCN, respectively) in male *Cntnap2* KO and matched wild type mice previously tested on an auditory battery. The MGN, VCN, and DCN were specifically chosen for stereological examination based on prior studies showing direct functional connectivity between the cochlear nucleus and MGN that allow for fast relay of auditory information, and ultimately efficient neural processing (Anderson et al., 2006). In the current experiment, numbers and sizes of neurons were estimated within the MGN, VCN, and DCN, respectively. In the MGN, we found significantly fewer neurons in *Cntnap2* KO mice. In addition, cumulative cell size distribution in the MGN indicated that *Cntnap2* KO mice had relatively more smaller and fewer larger neurons in the MGN as compared to congenic controls. Examination of the VCN and DCN yielded no differences in neuronal cell population or cell size distribution. These findings suggest that changes in MGN morphology may contribute to the atypical auditory processing behaviors observed in *Cntnap2* KO mice.

6.3 Methods and Materials

6.3.1 Subjects

Twelve male *Cntnap2* KO mice (B6.129(Cg)-*Cntnap*^{2tm1Pele}/J; stock number 017482) and 12 male wild type (WT) controls (C57BL/6J; stock number 000664) were obtained from The Jackson Laboratory (Bar Harbor, ME) and delivered to the University of Connecticut on postnatal day 49 (P49). Detailed descriptions of auditory processing paradigms conducted on these subjects as well as collected results were reported elsewhere (Truong et al., 2014). Briefly, subjects were assessed for their ability to detect silent "gaps" of variable duration (0–100 ms) within broad band white noise as well as a task that determined whether subjects were capable of detecting a variable duration change in frequency embedded within a different pure tone background (embedded tone 0–300 ms and embedded tone 0–100 ms). A pitch discrimination task was also conducted on the subjects.

6.3.2 Histology

Following end of behavioral testing (P150), subjects were weighed, anesthetized using a cocktail of ketamine (100 mg/kg) and xylazine (15 mg/kg), and transcardially perfused using a 0.9% saline solution followed by 4% paraformaldehyde. Brains were then removed from the skull, weighed, bottled, and post fixed in 4% paraformaldehyde. After at least 1 week of post fixation in 4% paraformaldehyde, brains were removed from the fixative and submerged in a cryoprotecting solution 24 hours prior to sectioning. Cryoprotected brains were serially sectioned in the coronal plane at 60 µm using a cryostat (Leica CM 3050S, Leica Biosystems, Buffalo Grove, IL). Every second section

was stained for nissl substance using cresyl violet, mounted on glass slides, and coverslipped using DPX mounting medium. Left and right hemispheres were distinquished by a notch carved above the left hemisphere in the celloidin during the embedding process.

6.3.3 Stereological measures

All prepared tissue were analyzed using Stereo Investigator (MBF Biosciences, Williston, VT, USA) integrated with a Zeiss Axio Imager A2 microscope (Carl Zeiss, Thornwood, NY). Experimenters were blind to subject genotype. Only neurons were assessed in the series of stereological experiments, and these were distinguished from non-neuronal cell types by the presence of a single distinct nucleolus within the cell nucleus. Neuronal cell populations were estimated using the optical fractionator probe with cross sectional neuronal cell area estimated concurrently using the nucleator probe. Neurons were only counted for analysis if the nucleolus was in focus and within the appropriate boundaries of the active counting frame and dissector depth. A standard stereotaxic atlas was used to determine the borders of the MGN, VCN, and DCN for quantification (Paxinos & Watson, 1986).

6.3.3.1 MGN cell measures

An average of 9 sections per brain contained the MGN and were used for analysis. Contours outlining the entirety of the MGN were drawn at 2.5X magnification. Cell size measurements and counts were assessed at 100X magnification. A sampling grid size of 225 µm × 225 µm and a 30 µm × 30 µm counting frame were used for stereological

examination. Estimates for both neuronal cell population and neuronal cell area were obtained for left MGN, right MGN, and total (left + right) MGN for each subject.

6.3.3.2 VCN and DCN cell measures

An average of 10 and 8 sections per brain contained the VCN and DCN, respectively, and these were used for analysis. Contours outlining the VCN and DCN (separately) were drawn at 2.5X magnification, with cell size measurements and population estimates obtained at 100X magnification. A sampling grid size of 225 µm × 225 µm and a 30 µm × 30 µm counting frame were used for stereological analysis. Estimates for both neuronal cell population and neuronal cell area were obtained for left, right, and total (left + right) VCN and left, right, and total (left + right) DCN.

6.3.4 Statistical Analysis

Mean cell count and mean cell size for MGN, VCN, and DCN were analyzed using a univariate ANOVA. Examination of cell size distribution was conducted using a cumulative percent distribution. To examine group differences in cell size distribution, nonparametric analyses using Mann-Whitney U and Kolmogorov Smirnov (K-S) tests were conducted on the cumulative percent distributions of each Genotype. All statistical analysis was conducted using SPSS 19 with an alpha criterion of $p<0.05$.

6.4 Results

6.4.1 Analysis of Hemisphere effects and normality

Initial examination of stereological data was conducted using left and right structures (MGN, VCN, DCN) separately, with Hemisphere as a within subject variable. Main

effects of Hemisphere, nor interactions, were found across neuronal cell population (Hemisphere, Hemisphere x Genotype, *p>0.05*) or mean cell size (Hemisphere, Hemisphere x Genotype, *p>0.05*). Therefore subsequent analyses collapsed left and right optical fractionator and nucleator data for each separate structure into a single score, reflecting total structure measures. Cumulative percent distributions of cell size for MGN, VCN, and DCN in *Cntnap2* KO and WT brains were separately tested for normality using a Shapiro-Wilk test. Results from this test across Structure and within each Genotype were significant (*p<0.001* for all measures), indicating that data did *not* follow a normal distribution. Therefore, analysis of cumulative cell size distribution was conducted using nonparametric analyses (Mann-Whitney U and K-S test).

6.4.2 Cntnap2 KO mice show reduced neuronal numbers and a cell size distribution shift toward smaller neurons in the MGN

A one-way ANOVA comparing total neuronal cell population in the MGN revealed a main effect of Genotype, indicating significantly fewer neurons in the MGN of *Cntnap2* KO mice in comparison to WT controls *[F(1,19)=5.35, p<0.05]* (Figure 6.1.A). Examination of mean cell size in the MGN found no main effect of Genotype *[F(1,19)<1, N.S.]* (Figure 6.1.B). However, comparisons of cumulative percent distributions between *Cntnap2* KO and WT controls using a Mann-Whitney U did revealed a significant difference in median cell size (*Cntnap2* KO Mdn = 97.96 μm^2; WT Mdn = 100.5 μm^2, *U = 9296863, p<0.001*), with follow-up analysis indicating a significant K-S statistic (*p<0.001*) (Figure 6.1.C). Taken together, analysis of cell size distribution in *Cntnap2* KO and WT brains revealed that the two distributions were

significantly different, with a leftward shift in cell size distribution toward smaller neurons in the MGN of Cntnap2 KO brains as compared to controls.

6.4.3 VCN and DCN show no changes in morphology in Cntnap2 KO brains

Comparison of total mean cell counts and mean cell size in the VCN showed no significant differences across Genotype (VCN cell count: *[F(1,19)<1, N.S.]*; VCN cell area: *[F(1,19)<1, N.S.]*; Figure 6.2.A–B). Nonparametric assessment of cell size distribution in the VCN also revealed no differences in median cell size or distribution (*Cntnap2* KO Mdn = 107.01 μm^2; WT Mdn = 107.71 μm^2, *U = 2210591, N.S.*; K-S test, *N.S.*; Figure 6.2.C). In the DCN, comparison of total mean cell counts and mean cell size further revealed no significant differences across Genotype (DCN cell count *[F(1,19)<1, N.S.]*; DCN cell area: *[F(1,19)=1.31, N.S.]*; Figure 6.3.A–B). Finally, null results were observed in the DCN, with no differences in median cell size or distribution (*Cntnap2* KO Mdn = 94.52 μm^2; WT Mdn = 96.43 μm^2, *U = 567174, N.S.*; K-S test, *N.S.*; Figure 6.3.C).

6.5 Discussion

Stereological analysis of subcortical structures associated with the central auditory pathway revealed significant differences in the cellular morphology of the MGN in *Cntnap2* KO mice as compared to their wild type controls. Specifically, we saw a significant reduction in the neuronal cell population in comparison to WTs. In addition, an examination of neuronal cell size (area) distribution between groups revealed a shift toward smaller neurons in the MGN of *Cntnap2* KO mice. No changes in cellular morphology (across estimated neuronal population, mean cell size, and cell size

distribution) were observed in the VCN or DCN among the two groups. Although structures in the cochlear nucleus showed no anatomical differences between groups, the observed differences in the MGN are fascinating in light of previous auditory behavioral evaluation conducted on this particular cohort of subjects, specifically showing atypical patterns of auditory processing (Truong et al., 2014).

Co-occurrence of these two findings across anatomy and behavior suggest that alterations in MGN morphology may potentially mediate or contribute to the abnormal auditory processing phenotype identified in *Cntnap2* KO mice. These findings together seem to correlate with independent observations related to generalized differences in thalamic structure (Cheon et al., 2011; Chugani et al., 1997; Friedman et al., 2003; Muller et al., 1998; Mizuno et al., 2006; Tamura et al., 2010; Tsatsanis et al., 2003) and atypical auditory processing behavior (both enhanced and deficient) in ASD populations (see O'Connor, 2012 and Ouimet et al., 2012, for review). Despite evidence of thalamic changes and atypical auditory processing findings in the clinical literature, few studies have been undertaken to ascertain functional links between changes in the thalamus (specifically MGN), and auditory processing anomalies. The series of experiments conducted by Roberts et al. (2011) are the most relevant, and thee studies examined potential relationships between structural changes related to the MGN and auditory processing. Here they measured the fractional anisotropy of projections emanating from the MGN—an index used to quantify myelination (i.e. fiber density, axonal diameter, etc.)—and also recorded auditory evoked neuro-magnetic fields in children with ASD. Although ASD subjects presented with a significant delay in the “middle latency” cortical 50 ms auditory response component (M50), no group differences were identified in white

matter fractional anisotropy projecting from the MGN. These findings, however, do not provide enough evidence to suggest that the MGN in general does not affect auditory sensory information, but it does show that changes in white matter density as measured by fractional anisotropy are not associated with a delayed M50 auditory response (Roberts et al., 2011). Therefore, additional structural and electrophysiological analysis must be conducted concurrently in ASD populations to directly evaluate the relationship between MGN morphology and auditory processing behavior in ASD.

Reductions in neuronal cell population in the MGN of *Cntnap2* KO mice also correspond to overall reductions in thalamic volume reported in the ASD literature (Tamura et al., 2010; Tsatsanis et al., 2003). No study has conducted *post mortem* histological analysis of brain tissue to examine neuronal cell population at the level of the thalamus in ASD individuals, but given the data obtained from *Cntnap2* KO mice, it may be that reductions in thalamic volume observed *in vivo* within the clinical population reflect a decrease in the number of cells within the structure. In the context of the *Cntnap2* KO stereological findings, it leads us to question why such changes are seen. It could be that mutation of *Cntnap2* may (directly or indirectly) lead to neuronal cell death in the thalamus, thus critically affecting neuronal cell number in the MGN. Alternatively, *Cntnap2* may play a role in neural proliferation early in cortical development. Should disruption in *Cntnap2* expression occur at this time, then a reduction in the number of neurons generated would be expected, ultimately leading to decreased neuronal cell populations. Interestingly, the CNTNAP2 protein shows embryonic expression in regions of neural proliferation including the ventricular zone (where neurons destined for the cerebral cortex are born) as well as the ganglionic eminences (where inhibitory

interneurons arise) in mice, suggesting a potential role in neuronal development (Peñagarikano et al., 2011). However, given the current data set, it is unclear whether cell death or disrupted neuronal proliferation explains the reduction in neuronal cell population in the MGN of *Cntnap2* KO brains. Future studies could address this issue in the brains of developing *Cntnap2* KO mice using Tunel (marker of cell death) or birthdating (e.g., BrdU) experiments. It is worth noting that previous assessment of *Cntnap2* KO brains did report a decrease in the number of GABAergic interneurons within all six layers of the cortex, striatum, and hippocampus (MGN was not examined; Peñagarikano et al., 2011), an affect associated with hyper-excitability. Therefore, it could be that the reduction we observed in neuronal cell population in the MGN reflects a specific decrease in inhibitory interneurons, and sparing of other (excitatory) neuronal cell types. However, histological preparation conducted in the present study simply stained for Nissl substance, which does not differentiate between cell types (glia, neurons, interneurons), nor excitatory versus inhibitory neurons. As such, additional experimentation should investigate this issue using cell-specific staining techniques targeting GABAergic interneurons.

Examination of the distribution of neuronal cell sizes in *Cntnap2* KO mice also found a significant leftward shift toward smaller neurons in the MGN. Unfortunately this finding does not have a correlate in the ASD literature, but these findings do parallel observations made in developmental dyslexia—another language-based neurodevelopmental disorder that may share some etiological components of language-related dysfunction with ASD (Frit, 1998; Rodenas-Cuadrado, Ho & Vernes, 2013). *Post mortem* histological analysis of dyslexic brains and rodent brains modeling

neuroanatomical features of dyslexia did reveal a striking shift in the cell size distribution of the MGN toward smaller cells (Galaburda, Menard & Rosen, 1994; Herman et al., 1997; Rosen et al., 2006; Rosen, Herman & Galaburda, 1999). Furthermore, neuromorphological changes in the MGN co-occur with auditory processing deficits in both dyslexic individuals, and also in rodent models of "dyslexia-like features" (Clark et al., 2000c; Cohen-Mimran & Sapir, 2007; Fitch et al., 1997; Fitch et al., 1994; Hari & Renvall, 2001; Peiffer, Rosen & Fitch, 2004; Peiffer et al., 2004; Tallal & Piercy, 1973; Vandermosten et al., 2011). Collectively, clinical and rodent literature focused on the study of dyslexia have verified a link between cellular changes in the MGN and atypical auditory processing behavior. Current findings in the brains of *Cntnap2* mice further support this association.

No changes in cellular morphology were observed in the VCN or DCN—structures that comprise the cochlear nucleus. Interestingly, in a case study using *post mortem* histological analysis of the brain from an autistic individual, no clear structural abnormalities in the cochlear nucleus were present (Rodier et al., 1996). Nonetheless, lack of atypical morphology in the cochlear nucleus of *Cntnap2* KO mice does not necessarily reflect "normal" functionality. Given that various electrophysiological studies conducted in ASD populations indicate dysfunction in auditory evoked responses at the level of the brainstem (e.g., using ABR paradigms), future study in *Cntnap2* KO mice should employ auditory processing experiments utilizing ABR techniques to determine whether deficits can be observed at low processing levels such as the auditory brainstem.

6.6 Conclusion

Previous auditory processing characterization of *Cntnap2* KO mice revealed an interesting dissociation invloving enhanced perception of pitch related information (pitch discrimination) and co-occuring impaired gap detection (silent gaps within broadbrand white noise). This is a pattern of atypical auditory processing behaviors observed in the ASD population. Given this abnormal auditory processing phenotype of *Cntnap2* KO mice, the purpose of the current study was to conduct a stereological assessment of subcortical structures along the central auditory pathway to determine whether a neural substrate could be identified to potentially explain the behavioral findings. Examining brains obtained from the previously reported auditory characterization study, stereological investigation revealed significant changes in the structural morphology of the MGN of mutants, but not in the VCN or DCN subdivisions of the cochlear nucleus. Specifically, reductions in neuronal cell population were observed in addition to a relative increase in the percentage of small cells in the MGN relative to large cells—thus representing an atypical cell size distribution as compared to WT brains. Together, these findings support an association between changes in MGN cellular morphology/organization, and atypical auditory processing behavior. Although the present anatomical analysis cannot empirically determine whether reduction in neuronal cell population or the shift to more small cells in the MGN directly disrupt auditory processing, results do provide a foundation for further examination of the role of the MGN (and other auditory related structures) in the manifestation of abnormal sensory processing in *Cntnap2* KO mice and in ASD. Results also expand upon the potential function and targets of disrupted *Cntnap2* expression in the brain. Clinically, *CNTNAP2*

shows great promise as a potential risk gene in ASD and SLI, specifically in the context of disrupted language development. Therefore, further investigation to elucidate the gene, brain, and behavior relationships of *CNTNAP2* is paramount in examining the underlying mechanisms associated with language disability, and ultimately the development of interventions and targets for therapy.

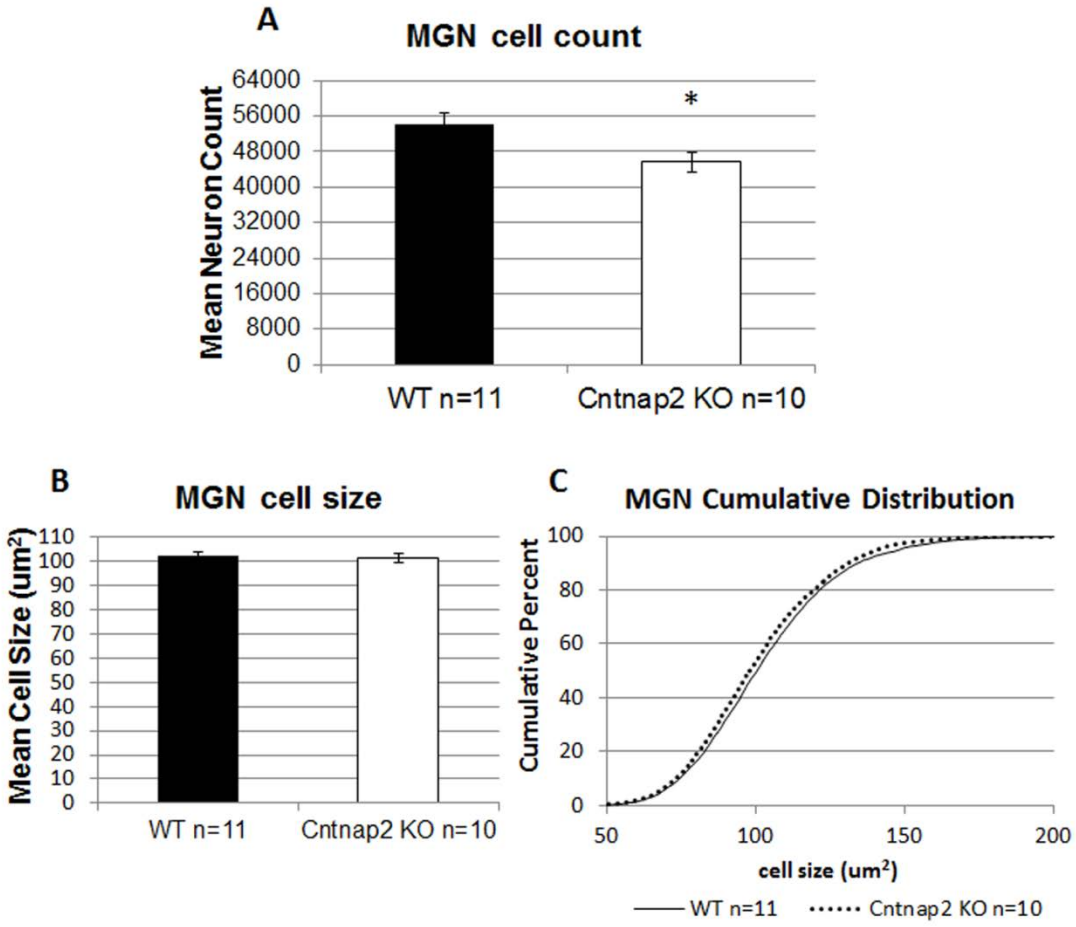

Figure 6.1. Stereological assessment of the medial geniculate nucleus (MGN). (A) Analysis of mean neuronal cell population in the MGN showed a significant reduction in the estimated total number of neurons in the MGN of *Cntnap2* KO mice in contrast to WT controls. (B) No differences in mean cell size were observed between groups. (C) However, comparison of cumulative percent distributions of neuronal cell size indicate a significant shift toward more small than large cells in the MGN of *Cntnap2* KO brains relative to controls. *P<0.05.

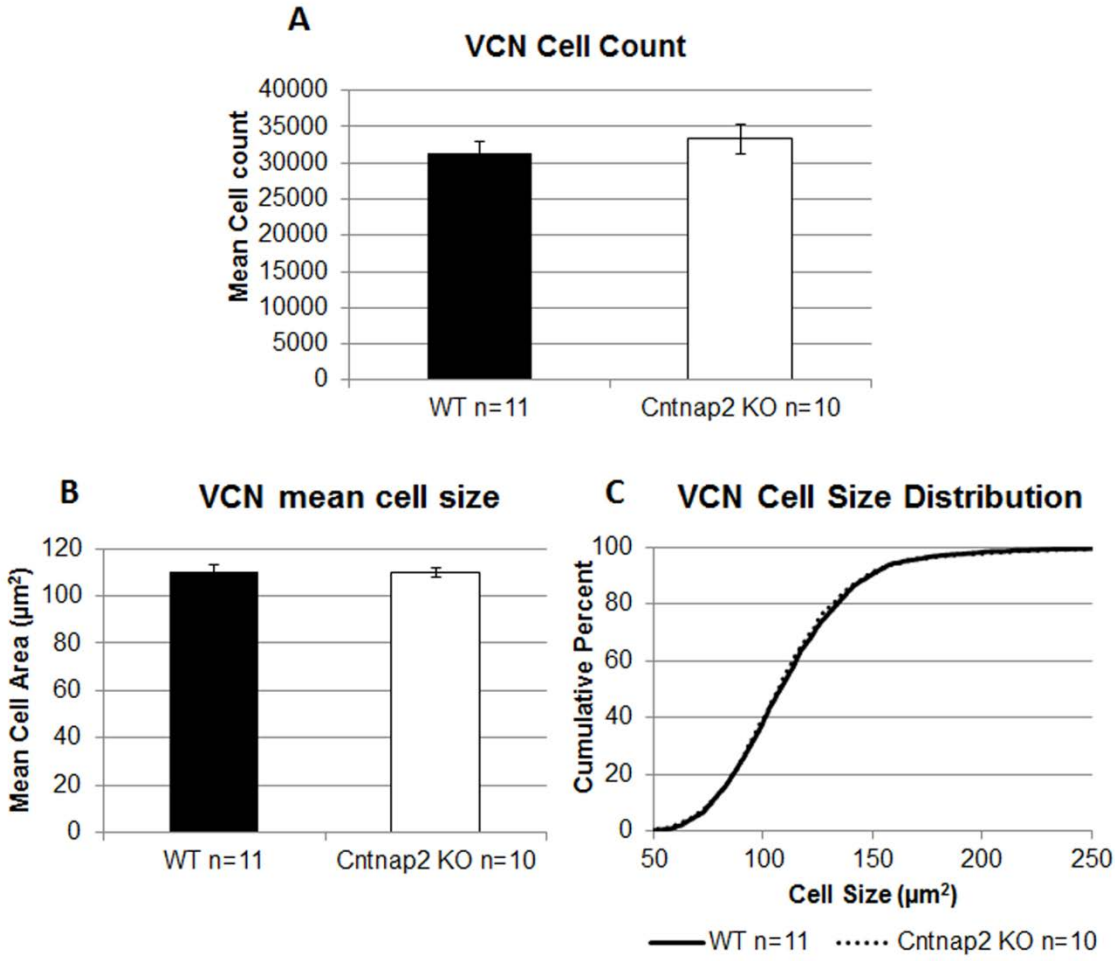

Figure 6.2. Stereological assessment of the ventral cochlear nucleus (VCN). (A) Estimates for neuronal cell population indicated no differences in neuronal cell population, (B) mean neuronal cell size, (C) or cumulative percent distribution of cell sizes between *Cntnap2* KO and WT brains.

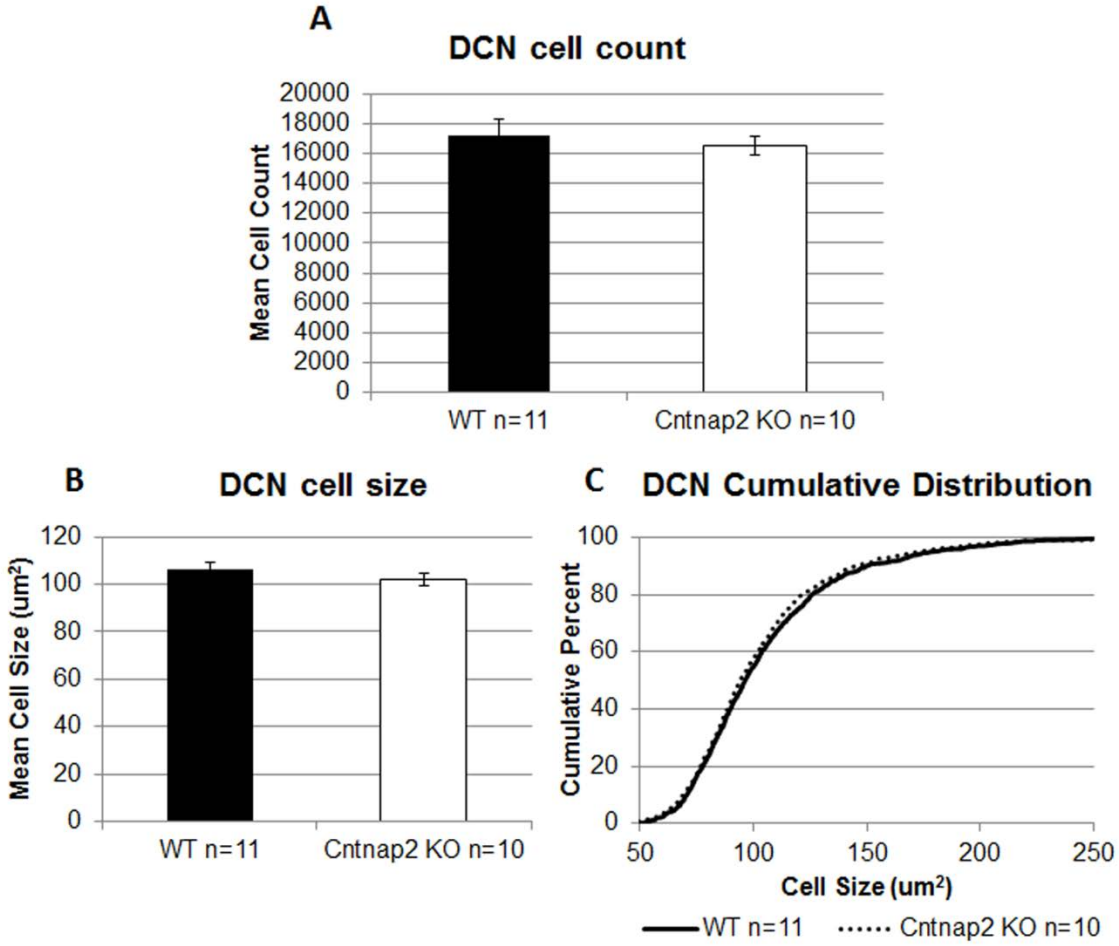

Figure 6.3. Stereological assessment of the dorsal cochlear nucleus (DCN). (A) Estimates for neuronal cell population indicated no differences in neuronal cell population, (B) mean neuronal cell size, (C) or cumulative percent distribution of cell sizes between *Cntnap2* KO and WT brains.

Table 7.1: Summary of BXD29- *Tlr4*$^{lps\text{-}2J}$/J findings (Chapters 2 and 3)

Silent Gap	Rotarod	Morris Water Maze	Social Adult Vocalizations	Social Preference	Stereology: MGN	Stereology: CN
+ Mutants (both sexes) worse	–	+ Female mutants better	+ Male mutants more vocalizations*	+ Mutants (both sexes), more social	+ Mutants (both sexes), cell size shift toward smaller neurons	+ Female mutants, cell size shift toward larger neurons

"+" indicates behavioral or neuroanatomical differences observed within that experiment.

"–" indicates both mutant and control groups showed similar phenotypes.

*Females were not tested on this task because they do not vocalize.

Table 7.2: Summary of *Dcdc2* KO findings (Chapter 4)

Silent Gap	Embedded Tone	4/8 radial arm water maze: Working memory	4/8 radial arm water maze: Reference memory	Rotarod	Tactile Discrimination
–	+ *Dcdc2* KO worse	+ *Dcdc2* KO worse	+ *Dcdc2* KO worse	–	+ *Dcdc2* KO worse

"+" indicates behavioral differences in performance between *Dcdc2* KO and WT controls.

"–" indicates similar performance on the task.

Table 7.3: Summary of *Cntnap2* KO findings (Chapters 5 and 6)

Silent Gap	Embedded Tone	Pitch Discrimination	Stereology: MGN	Stereology: CN
+ *Cntnap2* KO worse	+ *Cntnap2* KO better	+ *Cntnap2* KO Better	+ *Cntnap2* KO reduction in neural population and cell size shift toward smaller neurons	–

"+" indicates behavioral and neuroanatomical differences between *Cntnap2* KO and WT controls.

"–" indicates similar performance of neuroanatomical presentation.

CHAPTER 7

Discussion

7.1 Summary of findings

The etiological underpinnings of language-based neurodevelopmental disorders remain highly elusive. However, several breakthroughs in neurological science suggest that underlying disruptions in cortical development leading to subtle neuroanatomical anomalies and/or altered neural connectivity might serve not only as a neural "marker" for developmental language disorders, but may also play a functional role in the manifestation of language disability. Indeed, cortical development is an exceptionally complex process that is highly regulated by numerous genetic pathways, with even the slightest deviance leading to potentially devastating outcomes for the individual. Therefore, it is unsurprising that a number of promising risk genes that have been associated with language-based neurodevelopmental disorders (such as *DCDC2* and *CNTNAP2*) are also shown to be functionally implicated in early cortical development (Burbridge et al., 2008; Meng et al., 2005; Peñagarikano et al., 2011).

The collection of completed studies presented in this thesis provides important insight into the intricate relationship between genetic expression, neuroanatomical development, and behavioral outcomes in the context of language and reading related endophenotypes. This series of experiments utilized various mouse models to examine consequences from loss of function of key genes highly implicated in clinical populations with impaired language and reading ability, as well as models of altered neuronal migration—a known neural substrate of complex developmental language disorders. In

so doing, we established neurobehavioral profiles that should allow us to further extrapolate the gene, brain, and behavior interactions implicated in language dysfunction, with translation to the clinical population. Furthermore, these studies expand upon existing research linking atypical neuromorphology spanning the cortex and subcortical structures to co-occuring aberrant behaviors associated with auditory processing and other fundamental phenotypes implicated in typical language related function.

A summary of all behavioral findings within the three models investigated are displayed in Tables 7.1 (BXD29-*Tlr4*$^{lps\text{-}2J}$/J; Chapters 2–3), 7.2 (*Dcdc2* KO; Chapter 4); and 7.3 (*Cntnap2* KO; Chapters 5–6). Overall, these data suggest that underlying disruptions in cortical and subcortical development are causal to subsequent dysfunction in fundamental core behavior domains, as well as anomalies of anatomic structure that underlie language related disability. Although the present studies cannot speak to the specific (direct) causal links between the different experimental manipulations and associated outcomes, they do provide behavioral and neuroanatomical targets for more thorough investigation, as well as clues to potential neural mechanisms that could lead to behavioral dysfunction.

7.2 Neuroanatomical effects on auditory processing

Data regarding neural substrates for language disability range from collections of subtle layer 1 ectopias and microgyria in *post mortem* histological analysis of dyslexic individuals, to numerous *in vivo* neuroimaging studies examining brains of language impaired populations reporting structural (e.g., reductions in grey and white matter volume, periventricular nodular heterotopia, and polymicrogyria) as well as functional connectivity changes in language and reading relevant neural networks. From these

findings, it has become very clear that language disability is associated with underlying disruption of early cortical development and neural network organization (Boscariol et al., 2009; Brambati et al., 2004; Chang et al., 2005; Chang et al., 2007; Christodoulou et al., 2013; Clark & Plante, 1998; Darki et al., 2012; Evans et al., 2014; Hasan et al., 2012; Klingberg et al., 2000; Kronbichler et al., 2008; Niogi & McCandliss, 2006; Pugh et al., 2000; Pugh et al., 2013; Waiter et al., 2005; Wegiel et al., 2010). This view is supported by studies conducted by Benasich and colleagues, which reported a correlation between resting state gamma oscillatory activity (a 30–80 Hz signature of neural synchrony associated with higher order cognitive processes including language) and individual test scores of language and cognitive skills at 16, 24, and 36 months of age (Benasich et al., 2008). In addition, notable differences in gamma activity were found between young children with a family history of language impairment versus matched controls (Benasich et al., 2008). Follow-up assessment of these children revealed that differences in resting gamma power recorded at 24 and 36 months of age were able to reliably predict language and cognitive outcomes at 4 and 5 years of age (Gou, Choudhury & Benasich, 2011). Furthermore, reductions in gamma power and synchrony were associated with impaired rapid auditory processing (RAP) in language impaired children as measured on a two-tone oddball paradigm (Heim et al., 2011). These series of studies from Benasich and colleagues are of great significance, because they reveal that consistent underlying disruptions in neural organization (synchrony) are already present when language is just emerging in young children. Unfortunately, the origins of altered neural synchrony early in language development in the Benasich cohort remain unknown, but other related experiments in both clinical and rodent models may help in gaining insight.

As one example, *in vivo* imaging studies conducted with individuals exhibiting periventricular nodular heterotopia (PNH; a MCD involving un-migrated neurons clustered around the ventricular zone) have shown that the heterotopia are both structurally and functionally connected to immediate and distal regions of the cerebral cortex, as well as subcortical structures (Christodoulou et al., 2012). The presence of PNH has also been linked to dyslexia, and related studies using functional MRI during reading tasks have revealed that PNH are in fact integrated into neural circuits activated during reading in these subjects (Christodoulou et al., 2013). In rodent models of MCD (modeling neuropathological findings from dyslexic individuals), tracing studies have also shown disrupted neural connectivity, with fibers projecting from the malformation itself and making synaptic contacts with distal (i.e., inter-hemispheric and subcortical) regions of the brain (Jenner, Galaburda & Sherman, 2000; Rosen, Burstein & Galaburda, 2000). Electrophysiological analysis of neurons located in ectopic collections of mice with spontaneously occurring malformations also showed that mismigrated neurons were in fact functionally integrated into the neural network—receiving both excitatory and inhibitory synaptic input, sending projections to underlying cortex, and increasing the general excitability of the brain (Gabel & LoTurco, 2001; Gabel & LoTurco, 2002; Gabel, 2011). Thus it appears that neuroanatomical anomalies present within the system can actually exhibit atypical functional connectivity with local cortical regions, as well as more distant areas of the brain (for example through corticofugal projections).

In the context of BXD29-*Tlr4*$^{lps\text{-}2J}$/J and *Cntnap2* KO mice, it may be that the MCD (see Rosen et al., 2013 and Peñagarikano et al., 2011, respectively, for more in depth descriptions of MCD) present in these models could contain aberrant, yet

functional, connections with surrounding cortex, and this aberrant local activity may in turn disrupt the development of distal connectivity. Resulting abnormal organization of the cortex could manifest in the expression of atypical auditory processing behaviors in comparison to typical controls. Furthermore, MCD could lead to top-down propagational changes via cortico-thalamic connectivity, and this could ultimately lead to developmental morphological changes in the thalamus (and specifically the MGN), in turn mediating the altered auditory processing behaviors observed in BXD29-*Tlr4*$^{lps\text{-}2J}$/J and *Cntnap2* KO mice. Organizational changes in the MGN have in fact been observed in injury-induced models of cerebro-cortical microgyria, where physical injury to the cortical plate overlying somatosensory cortex cannot directly explain the altered MGN morphology observed (given that injury induced neural degeneration is expected, and is seen, but in the ventral posterior nucleus of the thalamus which projects directly to somatosensory cortex; Herman et al., 1997; Rosen et al., 2006). Therefore, it can further be argued that MCD in development could lead to atypical connectivity that could mediate the changes in both cell size distribution and general organization of the MGN, and may ultimately influence low level auditory processing. However, it is worth mentioning that the MCD observed in *Cntnap2* KO mice are very subtle, and explanations for the altered auditory processing phenotype and MGN morphology seen in this model are likely mediated by other additional factors related to neural connectivity deviations (discussed further below).

7.3 Genetic effects on auditory processing and other language-related endophenotypes

7.3.1 Dcdc2

RNAi induced genetic knockdown of *Dcdc2* in rats leads to diffuse disruption in cortical migration (Burbridge et al., 2008; Meng et al., 2005). However, neuroanatomical analysis of mice with a systemic KO of *Dcdc2* revealed no gross neuromorphological changes in the cortex (Wang et al., 2011). Despite the absence of gross MCD in *Dcdc2* KO mice, neurophysiologic characterization identified both increased spike rate and decreased temporal precision in action potential firing of layer 4 and 2/3 pyramidal neurons relative to WT, bringing to light dysfunction in neural connectivity at the level of the synapse (Che, Girgenti & LoTurco, 2013). Observations of generalized memory impairments across working and reference memory ability, RAP anomalies, and impairments in detecting tactile spatial frequency differences of sandpaper in *Dcdc2* KO mice, could in turn reflect alterations in neural spike timing and rate that could underlie this diffuse behavioral phenotype. As described earlier, work from the Benasich group also showed that disruptions in gamma band oscillatory activity (neural asynchrony) was associated with language impairment and atypical RAP in children (Heim et al., 2011). Another study specifically examining dyslexic children found unstable neural representations to speech sounds, with significant trial-by-trial variability in neural response to the same auditory stimulus (Hornickel & Kraus, 2013). Similarly, unstable (“noisy”) cortical responses to human speech sounds were observed in rats with a genetic knockdown of another candidate dyslexia risk gene, *Kiaa0319*. Other clinical studies have also shown altered neurophysiology and behavior across visual and somatosensory domains in

dyslexic children and adults, suggesting an underlying impairment in temporal processing across several sensory domains (Eden et al., 1995; Laasonen, Service & Virsu, 2001; Laasonen, Service & Virsu, 2002; Livingstone et al., 1991; Stein, 2001; Stoodley et al., 2000). In the case of memory ability, it is known that synchronous neural ensemble activity is necessary for consolidation of memory in the hippocampus (Axmacher et al., 2006; Fell et al., 2001). Based on the clinical and rodent literature, it seems that consistent and precise temporal encoding of initial sensory information may be necessary to for the development of accurate statistical learning and discrimination of discrete information. In the case of *Dcdc2* KO mice, sensory and memory impairments across cognitive, auditory, and tactile modalities could reflect inconsistent and dysfunctional neural population coding in the system.

7.3.2 Cntnap2

Although *Cntnap2* KO mice do show MCD, the subtle nature of these malformations make it unlikely that the ectopic collections of neurons found in subcortical white matter could represent the exclusive cause of the pervasive abnormalities observed across social, repetitive, communicative, and auditory processing behaviors (Peñagarikano et al., 2011). However, electrophysiological findings in *Cntnap2* KO mice also show aberrant neural synchrony, which may be mediated by the significant reduction in cortical GABAergic interneuron population (Peñagarikano et al., 2011). This relationship is crucial, since there is evidence suggesting the importance of GABAergic interneurons in regulating patterns of neural synchrony throughout the brain (Gonzalez-Burgos & Lewis, 2008; Mann & Paulsen, 2007). In sum, it is very likely that the broader neural phenotype of atypical neural synchrony observed in *Cntnap2* KO mice could mediate the differences

seen in auditory processing behavior. What remains unclear is how dysfunctional cortical synchrony can manifest into *both* enhanced *and* deficient auditory processing. Future studies using the *Cntnap2* model, and drawing more direct correlations between anatomic, physiologic and behavioral anomalies, will be needed to address this complex issue.

7.4 Future Studies

In the current thesis, genetic models examining loss-of-function of clinically relevant genes implicated in language disability, as well as a model of MCD, were utilized to examine behavioral consequences across paradigms designed to test core, non-verbal, "intermediate phenotypes" of language and reading disability. In these series of studies, we were successful in identifying a number of behavioral abnormalities associated with each experimental manipulation. However, with these discoveries, a new series of questions and experiments are generated that need to be addressed to better understand the potential etiological mechanisms associated with the observed behavioral outcomes.

7.4.1 Incorporating sex as a variable in all models to understand and identify sex specific mechanisms.

First and foremost, all future studies should utilize both male and female subjects. Considering the higher prevalence of males diagnosed with language-related neurodevelopmental disorders compared to females, more work is necessary to better understand the mechanisms underlying the apparent sex difference in incidence. However, it must not be ignored that females are also diagnosed with neurodevelopmental disorders (albeit at a lower incidence rate). To further complicate matters, it has been reported that despite having the same clinical diagnosis, males still

show a greater behavioral disadvantage (Liederman, Kantrowitz & Flannery, 2005). Examination of BXD29-*Tlr4*$^{lps\text{-}2J}$/J mice revealed no sex differences in impaired auditory processing. However sex specific differences in Morris water maze performance emerged, revealing a specific BXD29-*Tlr4*$^{lps\text{-}2J}$/J female advantage. In addition, only female BXD29-*Tlr4*$^{lps\text{-}2J}$/J mice, only, showed significant changes in the cellular morphology of neurons located in the cochlear nucleus (with larger neurons). The sexual dimorphism indicates that there may be sex specific mechanisms leading to neural reorganization following disruption of neuronal migration in BXD29-*Tlr4*$^{lps\text{-}2J}$/J mice. In addition to conducting tracing studies to visualize possible changes in structural connectivity relative to sex (further described below), one way to investigate whether observed sex differences are genetically or hormonally regulated could be conducted using a "four core genotype" model (see Arnold & Chen, 2009, for review). To conduct this experiment, selective breedings are conducted to allow scientists to isolate chromosomal sex from hormonal sex, and thus to empirically examine chromosomal versus hormonally mediated sex differences. If such experiments were to be made possible in BXD29-*Tlr4*$^{lps\text{-}2J}$/J mice, we may be able to determine if sexually dimorphic behaviors and neuroanatomy reported in the current thesis are regulated hormonally or genetically, thus providing a greater understanding of sex-specific mechanisms that are causal in the etiology of sex differences in the BXD29-*Tlr4*$^{lps\text{-}2J}$/J mice mouse model. Findings from such research could further provide insight into sex-specific therapeutic targets for both behavioral and pharmacological intervention.

7.4.2 BXD29-Tlr4$^{lps\text{-}2J}$/J mice—future Studies examining the functional etiology of behavioral abnormalities

The major behavioral findings observed in BXD29-*Tlr4*$^{lps\text{-}2J}$/J mice included a severe impairment in auditory processing as well as a hypersocial phenotype. Examination of subcortical neurpathological changes in BXD29-*Tlr4*$^{lps\text{-}2J}$/J mice also found overall smaller neurons in the MGN, along with a significant difference in cell size distribution revealing a greater population of small as compared to large neurons in the MGN. However, it can only be inferred that wide-spread changes across behavior and morphology are etiologically related to the diffuse MCD present in BXD29- *Tlr4*$^{lps\text{-}2J}$/J mice. Prior neuroanatomical tracing studies have in fact been conducted to examine potential functional connectivity between MCD, cortico-thalamic and thalamo-cortical, as well as other relevant regions of the cortex in a mouse model of spontaneous neural ectopias and injury-induced cortical microgyria (Jenner, Galaburda & Sherman, 2000; Rosen, Burstein & Galaburda, 2000). When compared to unaffected homotopic cortex, aberrations in structural connectivity were identified, suggesting that MCD altered underlying cortical "wiring." Interestingly, howere, not all models of MCD show atypical connectivity (at least as measured by neural tracing) from homotopic cortex. One example of this is the heterotopic cortex (HeCo) mutant mouse, which shows a similarly diffuse (but geographically different) spontaneously occurring MCD as compared to BXD29-*Tlr4*$^{lps\text{-}2J}$/J mice (Croquelois et al., 2009).

Alternatively, it may be possible that MCD, or the genetic mechanisms regulating atypical development in BXD29-*Tlr4*$^{lps\text{-}2J}$/J mice, are not directly associated with behavioral dysfunctions reported. It could be that other (genetic) mechanisms altering

behavioral function could be at play. Genetic backcrossing experiments conducted by Rosen et al. (2013) to determine the genetic transmission of MCD found that the pattern of inheritance followed a two-locus autosomal recessive model, with additional assessment indicating that a separate known mutation in the *Tlr4* gene of BXD29-*Tlr4*$^{lps-2J}$/J mice was *not* the casual factor. However, it remains unclear if genetic mutation of the *Tlr4* gene could mediate the severe auditory processing phenotype. Therefore, additional experiments should be conducted on other strains of mice with known *Tlr4* mutations (e.g., B6(Cg)-Tlr4tm1.1Karp/J, B6.B10ScN-Tlr4lps-del/JthJ) with behavioral assessment of auditory processing outcomes.

7.4.3 Dcdc2 KO mice—future Studies utilizing pharmacological interventions to elucidate functional etiology and assess amelioration of behavioral deficits

Due to the inconsistent spike timing phenotype observed in *Dcdc2* KO mice, a whole transcriptome RNA-seq was conducted by Che et al. (2013) to determine if changes in transcriptional expression of relevant ion channels and receptors could be identified between *Dcdc2* KO as compared to WTs. Interestingly, an up-regulation in *Grin2B* expression (a gene that encodes the 2B subunit of NMDA receptors) was found in *Dcdc2* KO mice. Follow-up electrophysiological analysis found that introduction of an NMDA receptor antagonist led to increased temporal precision in action potential firing, thus "rescuing" the phenotype in *Dcdc2* KO mice. Given this finding, it would be interesting in the future to examine whether an NMDA receptor blocker could also rescue the impaired RAP, memory, and tactile discrimination behaviors reported in the current thesis. Such a study would provide not only a direct therapeutic application, but would provide insight as to whether the aberrant synaptic properties of *Dcdc2* KO mice could

explain the observed behavioral impairments. Fortunately, there is already an FDA approved NMDA receptor blocker on the market, Memantine, that is currently being used to treat cognitive symptoms of Alzheimers. Through potential future research (both pre-clinical and clinical), this class of drug may ultimately have an alternate use as a potential pharmacological treatment for those with dyslexia.

7.4.4 Cntnap2 KO mice—future studies to determine presence of neurophysiologic substrates of co-occurring enhanced and deficient auditory processing behaviors

An intriguing co-occurrence of enhanced and impaired auditory processing behavior was found in *Cntnap2* KO mice—a pattern of atypical auditory processing behaviors also observed in ASD individuals. To better understand the neural mechanisms that may underlie this puzzling phenotype, electrophysiological experiments using ABR and AERP could be utilized to better understand how different levels of the auditory system (brainstem versus cortical) may differentially process auditory information, ranging from spectrotemporally complex acoustic information to simple tone discrimination in ASD. In fact, in the ASD literature, delayed and inconsistent ABRs are reported in response to the presentation of speech or rapid click stimuli (see O'Connor, 2012, for review). Interestingly, only one study has examined the relationship between ABR and pitch-related information in ASD populations, and this study specifically examined changes in ABR to prosaic changes in speech sounds. Greater variability in the ABR signal was observed in ASD individuals, reflecting impaired processing of prosody at the level of the brainstem (Russo et al., 2008). However, it is unclear whether accurate ABR would be observed with presentation of simple changes in pitch. At the neocortical level, reduced

latencies in the auditory mismatch negativity component are observed in ASD subjects during simple pitch processing—a signal that suggests more efficient auditory processing. However, latencies increase and the signal degrades when more aurally complex/speech information is used, also indicating inefficient or dysfunctional processing (see O'Connor, 2012, for review). Based on the clinical literature, an examination of electrophysiological signatures within different neural structures along the auditory pathway in response to simple pitch or complex auditory stimuli in ASD subjects would provide insight into the initial locus of atypical processing. Such an experiment would also provide information about how simple pitch and spectrotemporally complex auditory information are differentially processed in the auditory system in general.

7.5 Final concluding remarks

The series of experiments presented in this thesis were designed to provide greater insight to the gene, brain, and behavior relationships that underlie the biological and behavioral development of language-related neurodevelopmental disorders. Specifically, we sought to examine how genetic and neuropathological factors relevant to clinical populations could mediate not only behavioral outcomes related to auditory processing, cognitive, and sensory discrimination ability, but also how these factors could be associated with other morphological changes within in the brain. Our findings have elucidated links between genetic and neuropathologic changes and fundamental behaviors underlying language dysfunction, and it is our hope that our work provides a larger platform from which additional hypotheses could be generated and tested to improve our understanding of the biological etiology of developmental language dysfunction. Ultimately, this

research may lead to the development of more personalized medicine to better treat the underlying biology of neurodevelopmental disorders, as well as to provide early targeted behavioral interventions based on individual genetic and neuropathologic risk factors.

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